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(54) Title: C–TERMINAL REGION OF AGOUTI–RELATED TRANSCRIPT (ART) PROTEIN

(57) Abstract

Novel polypeptides derived from the C–terminal region of the human and mouse agouti related transcript (ART) proteins are provided. Also provided are DNA sequences encoding the novel C–terminal polypeptides. The novel C–terminal polypeptides can be used to inhibit the binding of melanocyte stimulating hormones to melanocortin receptors. Methods of identifying inhibitors of the binding of ART protein to melanocortin receptors are also provided.

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TITLE OF THE INVENTION

C-TERMINAL REGION OF AGOUTI-RELATED TRANSCRIPT (ART) PROTEIN

CROSS-REFERENCE TO RELATED APPLICATIONS

This application claims the benefit of U.S. Provisional Application No. 60/069,747, filed December 16, 1997, the contents of which are incorporated herein by reference in their entirety.

STATEMENT REGARDING FEDERALLY-SPONSORED R&D

Not applicable

REFERENCE TO MICROFICHE APPENDIX

Not applicable

FIELD OF THE INVENTION

The present invention is directed to polypeptides derived from the C-terminal region of the agouti-related transcript (ART) protein and to uses of such polypeptides, including use as inhibitors of the binding of melanocyte stimulating hormones to melanocortin receptors, and use to identify inhibitors of the binding of ART protein to melanocortin receptors.

BACKGROUND OF THE INVENTION

ART (agouti related transcript) was originally discovered as an mRNA that is upregulated in the hypothalamus of ob/ob and db/db mice. The ART gene has been cloned from both mice and humans and encodes a protein of 131 amino acids in mice and 132 amino acids in humans (Shutter *et al.*, 1997, Genes and Development 11:593-602). Recombinantly produced ART protein has been shown to be a functional antagonist of the melanocortin-3 receptor (MC3R) and the melanocortin-4 receptor (MC4R) (Fong *et al.*, 1997, Biochem. Biophys. Res. Comm. 237:629-631; Ollman *et al.*, 1997, Science 278:135-138).

MC3R and MC4R belong to a class of G-protein coupled receptors known as the melanocortin receptors, since these receptors activate adenylyl cyclase in response to ligands known as melanocortins (*e.g.*, adrenocorticotrophin (ACTH) and the α-, β-, and γ-melanocyte

stimulating hormones). MC3R and MC4R are neural melanocortin receptors, with MC3R being expressed in the hypothalamus and limbic system of the brain and MC4R being expressed widely in the brain. In particular, MC4R expression has been found in a number of hypothalamic sites, including the ventromedial, lateral, dorsomedial, and paraventricular nuclei (Mountjoy *et al.*, 1994, Mol. Endocrinol. 8:1298-1308), regions which have been shown to play a role in feeding behavior (Bray, 1987, Nutr. Rev. 45:33-43). Gene targeting experiments have shown that MC4R has an important role in the control of feeding behavior and obesity. Knockout mice lacking MC4R develop an obesity syndrome characterized by hyperphagia, hyperinsulinemia, and hyperglycemia (Huszar *et al.*, 1997, Cell 88:131-141).

In view of this, there is great interest in the ART protein, which appears to be a natural regulator of MC3R and MC4R in humans. It is believed that the ART protein is likely to be a natural regulator of human obesity which functions by antagonizing either MC3R or MC4R. Accordingly, the identification of substances that inhibit the binding of ART protein to MC3R or MC4R is desirable, since such inhibitors are likely to be of value in the control of obesity. Substances that potentiate the effect of ART protein on MC3R or MC4R are also likely to be of value in the control of body weight.

SUMMARY OF THE INVENTION

The present invention provides novel polypeptides derived from the C-terminal region of the human and mouse ART proteins. Also provided are DNA sequences encoding the novel C-terminal polypeptides. The novel C-terminal polypeptides can be used to inhibit the binding of melanocyte stimulating hormones to melanocortin receptors. Methods of identifying inhibitors of the effect of ART protein on the binding of melanocyte stimulating hormones to melanocortin receptors are also provided.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows the quantitation of c-ART-b from COS-7 cells through the use of an ELISA using an antibody that recognizes the myc epitope in c-ART-b. Shown is the standard curve generated using known amounts of myc peptide. For the c-ART-b preparation made

from COS-7 cells, a 10x dilution of the sample gave an absorbance of 0.079, corresponding to 10 nM in the above standard curve. Therefore, the c-ART-b preparation had a concentration of 100 nM.

Figure 2 shows the binding affinity of c-ART-b for the human MC3R. Shown is the inhibition of ^{125}I-[Tyr^2][Nle^4, D-Phe^7] α-melanocyte stimulating hormone (^{125}I-NDP-α-MSH) binding to the human MC3R by c-ART-b.

Figure 3 shows the binding affinity of c-ART-b for the human MC4R. Shown is the inhibition of ^{125}I-NDP-α-MSH binding to the human MC4R by c-ART-b.

DETAILED DESCRIPTION OF THE INVENTION

The present invention provides C-terminal polypeptides derived from the agouti-related transcript (ART) protein and DNA sequences encoding those polypeptides. The C-terminal polypeptides from the ART protein are referred to herein as "ART polypeptides". In certain embodiments, the ART polypeptides are present in a contiguous polypeptide sequence, *i.e.*, a fusion protein, that incorporates, generally at the C-terminus of the fusion protein, one or more amino acid sequences not derived from the ART protein. Such non-ART protein sequences can be, *e.g.*, "tags", such as a protein kinase A site (for easier radioisotope labeling) or an antigenic sequence (*e.g.*, a myc epitope) for ELISA quantitation. Other tags are known in the art and ART polypeptides incorporating such other tags are included in the present invention. In other embodiments, the ART polypeptides are present in a fusion protein with another protein that gives rise to an easily detectable signal, *e.g.*, alkaline phosphatase (ART-AP) or luciferase (ART-luc). Fusion proteins such as ART-AP or ART-luc are useful in binding assays since their presence and/or concentration can be detected without the use of radioactivity.

In particular, the present invention includes the following ART polypeptides:

c-ART-a: This polypeptide contains, from N to C terminus: (1) a yeast signal sequence peptide; (2) amino acids 76-132 of the human ART protein; (3) a thrombin site; (4) a myc epitope; (5) a

protein kinase A (PKA) site; and (6) a hexahistidine tag. The amino acid sequence of c-ART-a is:

MNIFYIFLFLLSFVQGLEHTHRRGSLVKRSSLQDREPRS
1
SRRCVRLHESCLGQQVPCCDPCATCYCRFFNAFCYCRKLG
2
TAMNPCSRTLVPRGSEQKLISEEDLNLRRASLGHHHHHH
3 4 5 6
(SEQ.ID.NO.:1)

c-ART-b: This polypeptide contains, from N to C terminus: (1) amino acids 1-26 of the human ART protein; (2) amino acids 76-132 of the human ART protein; (3) a thrombin site; (4) a myc epitope; and (5) a hexahistidine tag. The amino acid sequence of c-ART-b is:

MLTAALLSCALLLALPATRGAQMGLALQDREPRSSRRCVRL
1
HESCLGQQVPCCDPCATCYCRFFNAFCYCRKLGTAMNPCS
2
RTLVPRGSGSELGTKLGPEQKLISEEDLNSAVDHHHHHH
3 4 5
(SEQ.ID.NO.:2)

c-ART-c: This polypeptide contains, from N to C terminus: (1) amino acids 1-26 of the human ART protein; (2) amino acids 76-132 of the human ART protein; (3) a thrombin site; (4) a PKA site; (5) a myc epitope; and (6) a hexahistidine tag. The amino acid sequence of
c-ART-c is:

MLTAALLSCALLLALPATRGAQMGLALQDREPRSSRRCVRL

1

HESCLGQQVPCCDPCATCYCRFFNAFCYCRKLGTAMNPCSRT

2

LVPRGSGSLRRASLGKLEQKLISEEDLNSAVDHHHHHH

3 4 5 6

(SEQ.ID.NO.:3)

ART-AP: This polypeptide contains, from N to C terminus: (1) amino acids 1-132 of the human ART protein; (2) a thrombin site; (3) the alkaline phosphatase protein; (4) a myc epitope; and (5) a hexahistidine tag. The amino acid sequence of ART-AP is:

MLTAALLSCALLLALPATRGAQMGLAPMEGIRRPDQALLP

ELPGLGLRAPLKKTNAEQAEEDLLQEAQALAEVLDLQDRE

1

PRSSRRCVRLHESCLGQQVPCCDPCATCYCRFFNAFCYCR

KLGTAMNPCSRTLVPRGSGSIIPVEEENPDFWNRQAAEAL

2

GAAKKLQPAQTAAKNLIIFLGDGMGVSTVTAARILKGQKK

DKLGPETFLAMDRFPYVALSKTYSVDKHVPDSGATATAYL

CGVKGNFQTIGLSAAARFNQCNTTRGNEVISVMNRAKKAG

KSVGVVTTTRVQHASPAGAYAHTVNRNWYSDADVPASA

RQEGCQDIATQLISNMDIDVILGGGRKYMFPMGTPDPEY

PDDYSQGGTRLDGKNLVQEWLAKHQGARYVWNRTELM

QASLDPSVTHLMGLFEPGDMKYEIHRDSTLDPSLMEMTE

3

AALRLLSRNPRGFFLFVEGGRIDHGHHESRAYRALTETIM

FDDAIERAGQLTSEEDTLSLVTADHSHVFSFGGYPLRGSS

IFGLAPGKARDRKAYTVLLYGNGPGYVLKDGARPDVTES

ESGSPEYRQQSAVPLDGETHAGEDVAVFARGPQAHLVHG

VQEQTFIAHVMAFAACLEPYTACDLAPSAGTTDAAHPG

KLGPEQKLISEEDLNSAVDHHHHHH (SEQ.ID.NO.:4)

4 5

ART-luc: This polypeptide contains, from N to C terminus: (1) amino acids 1-132 of the human ART protein; (2) a thrombin site; (3) the luciferase protein; (4) a myc epitope; and (5) a hexahistidine tag. The amino acid sequence of ART-luc is:

MLTAALLSCALLLALPATRGAQMGLAPMEGIRRPDQALLP

ELPGLGLRAPLKKTNAEQAEEDLLQEAQALAEVLDLQDRE

1

PRSSRRCVRLHESCLGQQVPCCDPCATCYCRFFNAFCYCR

KLGTAMNPCSRTLVPRGSGSMSIENNILIGPPPYYPLEEG

2

TAGEQLHRAISRYAAVPGTLAYTDVHTELEVTYKEFLDVT

CRLAEAMKNYGLGLQHTISVCSENCVQFFMPICAALYVG

VATAPTNDIYNERELYNSLSISQPTVVFTSRNSLQKILGVQ

SRLPIIKKIIILDGKKDYLGYQSMQSFMKEHVPANFNVSA

FKPLSFDLDRVACIMNSSGSTGLPKGVPISHRNTIYRFSH

CRDPVFGNQIIPDTTILCAVPFHHAFGTFTNLGYLICGFH

VVLMYRFNEHLFLQTLQDYKCQSALLVPTVLAFLAKNPL
3
VDKYDLSNLHEIASGGAPLSKEISEIAAKRFKLPGIRQGYG
LTETTCAIVITAEGEFKLGAVGKVVPFYSLKVLDLNTGKK
LGPNERGEICFKGPMIMKGYINNPEATRELIDEEGWIHSG
DIGYFDEDGHVYIVDRLKSLIKYKGYQVPPAELEALLLQH
PFIEDAGVAGVPDEVAGDLPGAVVVLKEGKSITEKEIQDY
VAGQVTSSKKLRGGVEFVKEVPKGFTGKIDTRKIKEILIK
AQKGKSKSKAKLGPEQKLISEEDLNSAVDHHHHHH (SEQ.ID.NO.:5)
4 5

The present invention also includes an ART polypeptide containing amino acids 1-26 and 76-132 of the human ART protein, having the following polypeptide sequence:

MLTAALLSCALLLALPATRGAQMGLALQDREPRSSRRCVRL
HESCLGQQVPCCDPCATCYCRFFNAFCYCRKLGTAMNPCSRT
(SEQ.ID.NO.:6)

The present invention also includes an ART polypeptide containing amino acids 76-132 of the human ART protein, having the following polypeptide sequence:

LQDREPRSSRRCVRLHESCLGQQVPCCDPCATCYCRFFNAFCYCRKL
GTAMNPCSRT (SEQ.ID.NO.:7)

The present invention also includes an ART polypeptide containing amino acids 1-26 and 75-131 of the mouse ART protein, having the following polypeptide sequence:

MLTAMLLSCVLLLALPPTLGVQMGVAPQNRESRSPRRCVRL
HESCLGQQVPCCDPCATCYCRFFNAFCYCRKLGTAMNLCSRT
(SEQ.ID.NO.:8)

The present invention also includes an ART polypeptide containing amino acids 75-131 of the mouse ART protein, having the following polypeptide sequence:

PQNRESRSPRRCVRLHESCLGQQVPCCDPCATCYCRFFNAFCYCRKLGT
AMNLCSRT (SEQ.ID.NO.:9)

The present invention also includes an ART polypeptide having the following sequence, from N to C terminus: (1) a yeast signal sequence peptide; (2) amino acids 75-131 of the mouse ART protein; (3) a thrombin site; (4) a myc epitope; (5) a PKA site; and (6) a hexahistidine tag. The amino acid sequence of this ART polypeptide is:

<u>MNIFYIFLFLLSFVQGLEHTHRRGSLVKRSS</u>PQNRESRSPRRCVRL
1
HESCLGQQVPCCDPCATCYCRFFNAFCYCRKLGTAMNLCSRT
2
<u>LVPRGS</u>EQKLISEEDLN<u>LRRASLG</u>HHHHHH (SEQ.ID.NO.:10)
3 4 5 6

The present invention also includes an ART polypeptide having the following sequence, from N to C terminus: (1) amino acids 1-26 of the mouse ART protein; (2) amino acids 75-131 of the mouse ART protein; (3) a thrombin site; (4) a myc epitope; and (5) a hexahistidine tag. The amino acid sequence of this ART polypeptide is:

<u>MLTAMLLSCVLLLALPPTLGVQMGVA</u>PQNRESRSPRRCVRL
1
HESCLGQQVPCCDPCATCYCRFFNAFCYCRKLGTAMNLCSRT
2
<u>LVPRGS</u>EQKLISEEDLN<u>LRRASLS</u>HHHHHH (SEQ.ID.NO.:11)
3 4 5 6

The present invention also includes an ART polypeptide having the following sequence, from N to C terminus: (1) amino acids 1-26 of the mouse ART protein; (2) amino acids 75-131 of the mouse ART protein; (3) a thrombin site; (4) PKA site; (5) a myc epitope; and (6) a hexahistidine tag. The amino acid sequence of this ART polypeptide is:

MLTAMLLSCVLLLALPPTLGVQMGVAPQNRESRSPRRCVRL
1
HESCLGQQVPCCDPCATCYCRFFNAFCYCRKLGTAMNLCSRT
2
LVPRGSGSLRRASLGKLEQKLISEEDLNHHHHHH
3 4 5 6
(SEQ.ID.NO.:12)

The present invention also includes an ART polypeptide, having the following sequence, from N to C terminus: (1) amino acids 1-131 of the mouse ART protein; and (2) the alkaline phosphatase protein. The amino acid sequence of this polypeptide is:

MLTAMLLSCVLLLALPPTLGVQMGVAPLKGIRRPDQAL

FP EFPGLSLNGLKKTNADRAEEVLLQKAEALAEVLDP
1
QNRESRSPRRCVRLHESCLGQQVPCCDPCATCYCRFFN

AFCYCRKLGTAMNLCSRTIIPVEEENPDFWNRQAAEAL

GAAKKLQPAQTAAKNLIIFLGDGMGVSTVTAARILKGQKK

DKLGPETFLAMDRFPYVALSKTYSVDKHVPDSGATATAYL

CGVKGNFQTIGLSAAARFNQCNTTRGNEVISVMNRAKKAG

KSVGVVTTTRVQHASPAGAYAHTVNRNWYSDADVPASA

RQEGCQDIATQLISNMDIDVILGGGRKYMFPMGTPDPEY

PDDYSQGGTRLDGKNLVQEWLAKHQGARYVWNRTELM

QASLDPSVTHLMGLFEPGDMKYEIHRDSTLDPSLMEMTE

2

AALRLLSRNPRGFFLFVEGGRIDHGHHESRAYRALTETIM

FDDAIERAGQLTSEEDTLSLVTADHSHVFSFGGYPLRGSS

IFGLAPGKARDRKAYTVLLYGNGPGYVLKDGARPDVTES

ESGSPEYRQQSAVPLDGETHAGEDVAVFARGPQAHLVHG

VQEQTFIAHVMAFAACLEPYTACDLAPSAGTTDAAHPG

(SEQ.ID.NO.:13)

The present invention also includes an ART polypeptide, having the following sequence, from N to C terminus: (1) amino acids 1-131 of the mouse ART protein; and (2) the luciferase protein. The amino acid sequence of this polypeptide is:

MLTAMLLSCVLLLALPPTLGVQMGVAPLKGIRRPDQALFP

EFPGLSLNGLKKTNADRAEEVLLQKAEALAEVLDPQNRES

1

RSPRRCVRLHESCLGQQVPCCDPCATCYCRFFNAFCYCRK

LGTAMNLCSRTMSIENNILIGPPPYYPLEEGTAGEQLHR

AISRYAAVPGTLAYTDVHTELEVTYKEFLDVTCRLAEA

MKNYGLGLQHTISVCSENCVQFFMPICAALYVGVATAP

TNDIYNERELYNSLSISQPTVVFTSRNSLQKILGVQSR

LPIIKKIIILDGKKDYLGYQSMQSFMKEHVPANFNVSA

FKPLSFDLDRVACIMNSSGSTGLPKGVPISHRNTIYRFSH

CRDPVFGNQIIPDTTILCAVPFHHAFGTFTNLGYLICGFH

VVLMYRFNEHLFLQTLQDYKCQSALLVPTVLAFLAKNPL
2
VDKYDLSNLHEIASGGAPLSKEISEIAAKRFKLPGIRQGYG

LTETTCAIVITAEGEFKLGAVGKVVPFYSLKVLDLNTGKK

LGPNERGEICFKGPMIMKGYINNPEATRELIDEEGWIHSG

DIGYFDEDGHVYIVDRLKSLIKYKGYQVPPAELEALLLQH

PFIEDAGVAGVPDEVAGDLPGAVVVLKEGKSITEKEIQDY

VAGQVTSSKKLRGGVEFVKEVPKGFTGKIDTRKIKEILIK

AQKGKSKSKAKL (SEQ.ID.NO.:14)

The present invention also includes an ART polypeptide, having the following sequence, from N to C terminus: (1) amino acids 1-26 of the human ART protein; (2) amino acids 76-132 of the human ART protein; (3) a thrombin site; (4) the alkaline phosphatase protein; (5) a myc epitope; and (6) a hexahistidine tag. The amino acid sequence of this polypeptide is:

MLTAALLSCALLLALPATRGAQMGLALQDREPRSSRRCV
1
RLHESCLGQQVPCCDPCATCYCRFFNAFCYCRKLGTA
2
MNPCSRTLVPRGSGSIIPVEEENPDFWNRQAAEAL
3
GAAKKLQPAQTAAKNLIIFLGDGMGVSTVTAARILKGQKK

DKLGPETFLAMDRFPYVALSKTYSVDKHVPDSGATATAYL

CGVKGNFQTIGLSAAARFNQCNTTRGNEVISVMNRAKKAG

KSVGVVTTTRVQHASPAGAYAHTVNRNWYSDADVPASA

RQEGCQDIATQLISNMDIDVILGGGRKYMFPMGTPDPEY

PDDYSQGGTRLDGKNLVQEWLAKHQGARYVWNRTELM

QASLDPSVTHLMGLFEPGDMKYEIHRDSTLDPSLMEMTE

4

AALRLLSRNPRGFFLFVEGGRIDHGHHESRAYRALTETIM

FDDAIERAGQLTSEEDTLSLVTADHSHVFSFGGYPLRGSS

IFGLAPGKARDRKAYTVLLYGNGPGYVLKDGARPDVTES

ESGSPEYRQQSAVPLDGETHAGEDVAVFARGPQAHLVHG

VQEQTFIAHVMAFAACLEPYTACDLAPSAGTTDAAHPG

KLGPEQKLISEEDLNSAVDHHHHHH (SEQ.ID.NO.:15)

5 6

The present invention also includes an ART polypeptide, having the following sequence, from N to C terminus, (1) amino acids 1-26 of the human ART protein; (2) amino acids 76-132 of the human ART protein; (3) a thrombin site; (4) the luciferase protein; (5) a myc epitope; and (6) a hexahistidine tag. The amino acid sequence of this polypeptide is:

MLTAALLSCALLLALPATRGAQMGLALQDREPRSSRRC

1

VRLHESCLGQQVPCCDPCATCYCRFFNAFCYCRKLGTA

2

MNPCSRTLVPRGSGSMSIENNILIGPPPYYPLEEGTAGEQLHR

3

AISRYAAVPGTLAYTDVHTELEVTYKEFLDVTCRLAEA

MKNYGLGLQHTISVCSENCVQFFMPICAALYVGVATAP

TNDIYNERELYNSLSISQPTVVFTSRNSLQKILGVQSR

LPIIKKIIILDGKKDYLGYQSMQSFMKEHVPANFNVSA

FKPLSFDLDRVACIMNSSGSTGLPKGVPISHRNTIYRFSH

CRDPVFGNQIIPDTTILCAVPFHHAFGTFTNLGYLICGFH

VVLMYRFNEHLFLQTLQDYKCQSALLVPTVLAFLAKNPL

4

VDKYDLSNLHEIASGGAPLSKEISEIAAKRFKLPGIRQGYG

LTETTCAIVITAEGEFKLGAVGKVVPFYSLKVLDLNTGKK

LGPNERGEICFKGPMIMKGYINNPEATRELIDEEGWIHSG

DIGYFDEDGHVYIVDRLKSLIKYKGYQVPPAELEALLLQH

PFIEDAGVAGVPDEVAGDLPGAVVVLKEGKSITEKEIQDY

VAGQVTSSKKLRGGVEFVKEVPKGFTGKIDTRKIKEILIK

AQKGKSKSKAKLGPEQKLISEEDLNSAVDHHHHHH (SEQ.ID.NO.:16)

5 6

The present invention also includes an ART polypeptide having the following sequence, from N to C terminus: (1) amino acids 1-132 of the human ART protein; and (2) the alkaline phosphatase protein. The amino acid sequence of this polypeptide is:

MLTAALLSCALLLALPATRGAQMGLAPMEGIRRPDQALLP

ELPGLGLRAPLKKTNAEQAEEDLLQEAQALAEVLDLQDRE

1

PRSSRRCVRLHESCLGQQVPCCDPCATCYCRFFNAFCYCR

KLGTAMNPCSRTIIPVEEENPDFWNRQAAEALGAAKKLQPA

QTAAKNLIIFLGDGMGVSTVTAARILKGQKKDKLGPETF

LAMDRFPYVALSKTYSVDKHVPDSGATATAYLCGVKGN

FQTIGLSAAARFNQCNTTRGNEVISVMNRAKKAGKSVG

VVTTTRVQHASPAGAYAHTVNRNWYSDADVPASARQ

EGCQDIATQLISNMDIDVILGGGRKYMFPMGTPDPEY

PDDYSQGGTRLDGKNLVQEWLAKHQGARYVWNRTELM

QASLDPSVTHLMGLFEPGDMKYEIHRDSTLDPSLMEMTE

2

AALRLLSRNPRGFFLFVEGGRIDHGHHESRAYRALTETIM

FDDAIERAGQLTSEEDTLSLVTADHSHVFSFGGYPLRGSS

IFGLAPGKARDRKAYTVLLYGNGPGYVLKDGARPDVTES

ESGSPEYRQQSAVPLDGETHAGEDVAVFARGPQAHLVHG

VQEQTFIAHVMAFAACLEPYTACDLAPSAGTTDAAHPG

(SEQ.ID.NO.:17)

The present invention also includes an ART polypeptide having the following sequence, from N to C terminus: (1) amino acids 1-132 of the human ART protein; and (2) the luciferase protein. The amino acid sequence of this polypeptide is:

MLTAALLSCALLLALPATRGAQMGLAPMEGIRRPDQALLP
ELPGLGLRAPLKKTNAEQAEEDLLQEAQALAEVLDLQDRE
1
PRSSRRCVRLHESCLGQQVPCCDPCATCYCRFFNAFCYCR
KLGTAMNPCSRTMSIENNILIGPPPYYPLEEGTAGEQLH
RAISRYAAVPGTLAYTDVHTELEVTYKEFLDVTCRLAE
AMKNYGLGLQHTISVCSENCVQFFMPICAALYVGVAT
APTNDIYNERELYNSLSISQPTVVFTSRNSLQKILGVQ
SRLPIIKKIIILDGKKDYLGYQSMQSFMKEHVPANFNVSA
FKPLSFDLDRVACIMNSSGSTGLPKGVPISHRNTIYRFSH
CRDPVFGNQIIPDTTILCAVPFHHAFGTFTNLGYLICGFH
VVLMYRFNEHLFLQTLQDYKCQSALLVPTVLAFLAKNPL
2
VDKYDLSNLHEIASGGAPLSKEISEIAAKRFKLPGIRQGYG
LTETTCAIVITAEGEFKLGAVGKVVPFYSLKVLDLNTGKK
LGPNERGEICFKGPMIMKGYINNPEATRELIDEEGWIHSG
DIGYFDEDGHVYIVDRLKSLIKYKGYQVPPAELEALLLQH
PFIEDAGVAGVPDEVAGDLPGAVVVLKEGKSITEKEIQDY
VAGQVTSSKKLRGGVEFVKEVPKGFTGKIDTRKIKEILIK
AQKGKSKSKAKL (SEQ.ID.NO.:18)

The present invention also includes an ART polypeptide having the following sequence, from N to C terminus: (1) amino acids 1-26 of the human ART protein; (2) the alkaline phosphatase protein; (3) amino acids 27-132 of the human ART protein. The amino acid sequence is:

MLTAALLSCALLLALPATRGAQMGLAIIPVEEENPDFWNRQAAEAL
1

GAAKKLQPAQTAAKNLIIFLGDGMGVSTVTAARILKGQKK

DKLGPETFLAMDRFPYVALSKTYSVDKHVPDSGATATAYL

CGVKGNFQTIGLSAAARFNQCNTTRGNEVISVMNRAKKAG

KSVGVVTTTRVQHASPAGAYAHTVNRNWYSDADVPASA

RQEGCQDIATQLISNMDIDVILGGGRKYMFPMGTPDPEY

PDDYSQGGTRLDGKNLVQEWLAKHQGARYVWNRTELM

QASLDPSVTHLMGLFEPGDMKYEIHRDSTLDPSLMEMTE
2

AALRLLSRNPRGFFLFVEGGRIDHGHHESRAYRALTETIM

FDDAIERAGQLTSEEDTLSLVTADHSHVFSFGGYPLRGSS

IFGLAPGKARDRKAYTVLLYGNGPGYVLKDGARPDVTES

ESGSPEYRQQSAVPLDGETHAGEDVAVFARGPQAHLVHG

VQEQTFIAHVMAFAACLEPYTACDLAPSAGTTDAAHPG

PMEGIRRPDQALLPELPGLGLRAPLKKTNAEQAEEDLLQE
3

AQALAEVLDLQDREPRSSRRCVRLHESCLGQQVPCCDPC

ATCYCRFFNAFCYCRKLGTAMNPCSRT (SEQ.ID.NO.:19)

The present invention also includes an ART polypeptide having the following sequence, from N to C terminus: (1) amino acids 1-26 of the human ART protein; (2) the luciferase protein; (3) amino acids 27-132 of the human ART protein. The amino acid sequence is:

MLTAALLSCALLLALPATRGAQMGLAMSIENNILIGPPPYYPLEEG

1

TAGEQLHRAISRYAAVPGTLAYTDVHTELEVTYKEFLDVT

CRLAEAMKNYGLGLQHTISVCSENCVQFFMPICAALYVG

VATAPTNDIYNERELYNSLSISQPTVVFTSRNSLQKILGVQ

SRLPIIKKIIILDGKKDYLGYQSMQSFMKEHVPANFNVSA

FKPLSFDLDRVACIMNSSGSTGLPKGVPISHRNTIYRFSH

CRDPVFGNQIIPDTTILCAVPFHHAFGTFTNLGYLICGFH

VVLMYRFNEHLFLQTLQDYKCQSALLVPTVLAFLAKNPL

2

VDKYDLSNLHEIASGGAPLSKEISEIAAKRFKLPGIRQGYG

LTETTCAIVITAEGEFKLGAVGKVVPFYSLKVLDLNTGKK

LGPNERGEICFKGPMIMKGYINNPEATRELIDEEGWIHSG

DIGYFDEDGHVYIVDRLKSLIKYKGYQVPPAELEALLLQH

PFIEDAGVAGVPDEVAGDLPGAVVVLKEGKSITEKEIQDY

VAGQVTSSKKLRGGVEFVKEVPKGFTGKIDTRKIKEILIK

AQKGKSKSKAKLPMEGIRRPDQALLPELPGLGLRAPLKK

TNAEQAEEDLLQEAQALAEVLDLQDREPRSSRRCVRLHE
3
SCLGQQVPCCDPCATCYCRFFNAFCYCRKLGTAMNPCSRT

(SEQ.ID.NO.:20)

The ART polypeptides of the present invention can be in a form that is substantially free from other polypeptides. "Substantially free from other polypeptides" means at least 90%, preferably 95%, more preferably 99%, and even more preferably 99.9%, free of other proteins. Thus, an ART polypeptide preparation that is substantially free from other polypeptides will contain, as a percent of its total polypeptides, no more than 10%, preferably no more than 5%, more preferably no more than 1%, and even more preferably no more than 0.1%, of non-ART polypeptides. Whether a given ART polypeptide preparation is substantially free from other polypeptides can be determined by such conventional techniques as, *e.g.*, sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) combined with appropriate staining methods, *e.g.*, silver staining.

It is possible to modify many of the amino acids of the ART polypeptides of the present invention and still retain substantially the same biological activity as possessed by the unmodified ART polypeptide. A modified ART polypeptide has "substantially the same biological activity" as an unmodified ART polypeptide if the modified polypeptide has an IC_{50} value for the inhibition of ^{125}I-labeled NDP-α-MSH binding to MC3R or MC4R that is no more than 5-fold greater than the IC_{50} value of the unmodified ART polypeptide for the inhibition of ^{125}I-labeled NDP-α-MSH binding to MC3R or MC4R.

Thus the present invention includes modified ART polypeptides which have amino acid deletions, additions, or substitutions but that still retain substantially the same biological activity as the unmodified ART polypeptide from which they are derived. It is generally accepted that single amino acid substitutions at non-critical positions do not usually alter the biological activity of a protein or polypeptide (see, *e.g.*, Molecular Biology of the Gene, Watson *et al.*, 1987, Fourth Ed., The Benjamin/Cummings Publishing Co., Inc., page 226; and Cunningham & Wells, 1989, Science 244:1081-1085). Accordingly,

the present invention includes modified polypeptides where one amino acid substitution has been made in SEQ.ID.NOs.:1-20 wherein the modified polypeptides still retain substantially the same biological activity as the unmodified ART polypeptides. The present invention also includes modified polypeptides where two amino acid substitutions have been made in SEQ.ID.NOs.:1-20 wherein the polypeptides still retain substantially the same biological activity as the unmodified ART polypeptides. More generally, the present invention includes modified polypeptides where amino acid substitutions have been made in regions of the polypeptides that are not critical, *i.e.*, in regions where modifications result in a polypeptide with substantially the same biological activity as the unmodified polypeptide.

In particular, the present invention includes embodiments where the above-described substitutions are conservative substitutions. A "conservative amino acid substitution" refers to the replacement of one amino acid residue by another, chemically similar, amino acid residue. Examples of such conservative substitutions are: substitution of one hydrophobic residue (isoleucine, leucine, valine, or methionine) for another; substitution of one polar residue for another polar residue of the same charge (*e.g.*, arginine for lysine; glutamic acid for aspartic acid).

The present invention also includes DNA sequences encoding polypeptides having the amino acid sequences of SEQ.ID.NOs.:1-20, with the proviso that, In the case of the DNA sequences encoding SEQ.ID.NOs.:6-9, the DNA sequences do not encode any contiguous stretch of amino acids from the ART protein other than SEQ.ID.NOs.:6-9.

The DNA sequences of the present invention can be in a form that is substantially free from other nucleic acids. "Substantially free from other nucleic acids" means at least 90%, preferably 95%, more preferably 99%, and even more preferably 99.9%, free of other nucleic acids. Thus, a preparation of DNA sequences encoding an ART polypeptide that is substantially free from other nucleic acids will contain, as a percent of its total nucleic acids, no more than 10%, preferably no more than 5%, more preferably no more than 1%, and even more preferably no more than 0.1%, of nucleic acids other than the DNA sequences encoding ART polypeptides. Whether a given preparation of

DNA sequences encoding an ART polypeptide is substantially free from other nucleic acids can be determined by such conventional techniques as, *e.g.*, agarose gel electrophoresis combined with appropriate staining methods, *e.g.*, ethidium bromide staining.

The DNA sequences of the present invention encoding ART polypeptides can be linked with other DNA sequences, *e.g.*, DNA sequences to which DNA sequences encoding the ART protein are not naturally linked, to form "recombinant DNA molecules" encoding ART polypeptides. Such other sequences can include DNA sequences that control transcription or translation such as, *e.g.*, translation initiation sequences, promoters for RNA polymerase II, transcription or translation termination sequences, enhancer sequences, sequences that control replication in microorganisms, or that confer antibiotic resistance. The DNA sequences of the present invention can be inserted into vectors such as plasmids, cosmids, viral vectors, or yeast artificial chromosomes.

Included in the present invention are DNA sequences that hybridize to the DNA sequences encoding ART polypeptides under stringent conditions. By way of example and not limitation, a procedure using conditions of high stringency is as follows: Prehybridization of filters containing DNA is carried out for 2 hr. to overnight at 65°C in buffer composed of 6X SSC, 5X Denhardt's solution, and 100 μg/ml denatured salmon sperm DNA. Filters are hybridized for 12 to 48 hrs at 65°C in prehybridization mixture containing 100 μg/ml denatured salmon sperm DNA and 5-20 X 10^6 cpm of ^{32}P-labeled probe. Washing of filters is done at 37°C for 1 hr in a solution containing 2X SSC, 0.1% SDS. This is followed by a wash in 0.1X SSC, 0.1% SDS at 50°C for 45 min. before autoradiography. Other procedures using conditions of high stringency would include either a hybridization carried out in 5XSSC, 5X Denhardt's solution, 50% formamide at 42°C for 12 to 48 hours or a washing step carried out in 0.2X SSPE, 0.2% SDS at 65°C for 30 to 60 minutes.

Reagents mentioned in the foregoing procedures for carrying out high stringency hybridization are well known in the art. Details of the composition of these reagents can be found in, *e.g.*, Sambrook, Fritsch, and Maniatis, 1989, Molecular Cloning: A Laboratory Manual, second edition, Cold Spring Harbor Laboratory

Press. In addition to the foregoing, other conditions of high stringency which may be used are well known in the art.

Another aspect of the present invention includes host cells that have been engineered to contain and/or express DNA sequences encoding ART polypeptides. Such recombinant host cells can be cultured under suitable conditions to produce ART polypeptides. An expression vector containing DNA encoding ART polypeptides can be used for expression of ART polypeptides in a recombinant host cell. Recombinant host cells may be prokaryotic or eukaryotic, including but not limited to, bacteria such as *E. coli*, fungal cells such as yeast, mammalian cells including, but not limited to, cell lines of human, bovine, porcine, monkey and rodent origin, and insect cells including but not limited to *Drosophila* and silkworm derived cell lines. Cell lines derived from mammalian species which are suitable for recombinant expression of ART polypeptides and which are commercially available, include but are not limited to, L cells L-M(TK$^-$) (ATCC CCL 1.3), L cells L-M (ATCC CCL 1.2), 293 (ATCC CRL 1573), Raji (ATCC CCL 86), CV-1 (ATCC CCL 70), COS-1 (ATCC CRL 1650), COS-7 (ATCC CRL 1651), CHO-K1 (ATCC CCL 61), 3T3 (ATCC CCL 92), NIH/3T3 (ATCC CRL 1658), HeLa (ATCC CCL 2), C127I (ATCC CRL 1616), BS-C-1 (ATCC CCL 26) and MRC-5 (ATCC CCL 171).

A variety of mammalian expression vectors can be used to express ART polypeptides in mammalian cells. Commercially available mammalian expression vectors which are suitable include, but are not limited to, pMC1neo (Stratagene), pSG5 (Stratagene), pcDNAI and pcDNAIamp, pcDNA3, pcDNA3.1, pCR3.1 (Invitrogen), EBO-pSV2-neo (ATCC 37593), pBPV-1(8-2) (ATCC 37110), pdBPV-MMTneo(342-12) (ATCC 37224), pRSVgpt (ATCC 37199), pRSVneo (ATCC 37198), and pSV2-dhfr (ATCC 37146). Following expression in recombinant cells, ART polypeptides can be purified by conventional techniques to a level that is substantially free from other proteins.

The present invention includes a method of determining whether a substance is an inhibitor of the binding of an ART polypeptide to a melanocortin receptor. Such substances are likely to be useful in the control of body weight. The method takes advantage of the fact that ART polypeptides inhibit the binding of melanocyte stimulating hormones to melanocortin receptors by themselves binding to the receptor. Thus, a

substance that antagonizes the inhibitory effect of ART polypeptides on the binding of melanocyte stimulating hormones to melanocortin receptors is likely to act by inhibiting the binding of the ART polypeptide itself to the melanoncortin receptor. The method comprises:

(a) providing cells expressing the melanocortin receptor;

(b) exposing the cells to a chosen concentration of the melanocyte stimulating hormone in the absence of the ART polypeptide and in the absence of the substance and measuring the amount of melanocyte stimulating hormone binding to the cells to obtain a first value for melanocyte stimulating hormone binding;

(c) exposing the cells to the chosen concentration of melanocyte stimulating hormone in the presence of a chosen concentration of the ART polypeptide and in the absence of the substance and measuring the amount of melanocyte stimulating hormone binding to obtain a second value for melanocyte stimulating hormone binding where the second value for melanocyte stimulating hormone binding indicates that less melanocyte stimulating hormone binding has occurred as compared to the first value for melanocyte stimulating hormone binding;

(d) exposing the cells to the chosen concentration of melanocyte stimulating hormone in the presence of the chosen concentration of ART polypeptide and in the presence of the substance and measuring the amount of melanocyte stimulating hormone binding to obtain a third value for melanocyte stimulating hormone binding;

where, if the third value for melanocyte stimulating hormone binding is greater than the second value, then the substance is an inhibitor of the binding of the ART polypeptide to the melanocortin receptor.

In a particular embodiment, the cells expressing the melanocortin receptor are cells that naturally express the melanocortin receptor. In another embodiment, the cells expressing the melanocortin receptor do not naturally express the melanocortin receptor but have been transfected with an expression vector that directs the expression of the melanocortin receptor. Transfection is meant to include any method known in the art for the introduction of the the expression vector directing the expression of the melanocortin receptor into the cells. For example, transfection includes calcium phosphate or calcium chloride

mediated transfection, lipofection, infection with a retroviral construct containing the melanocortin receptor, and electroporation.

In a particular embodiment, the melanocortin receptor is selected from the group consisting of: the melanocortin-3 receptor (MC3R) and the melanocortin-4 receptor (MC4R). In a particular embodiment of the above-described method, the melanocortin receptor is not a *Xenopus* melanocortin receptor.

The cells that have been transfected with an expression vector that directs the expression of the melanocortin receptor can be prokaryotic cells or eukaryotic cells. In a particular embodiment, the cells that have been transfected with an expression vector that directs the expression of the melanocortin receptor are selected from the group consisting of: yeast cells, mammalian cells, bacterial cells, and insect cells. In a particular embodiment, the cells that have been transfected with an expression vector that directs the expression of the melanocortin receptor are selected from the group consisting of: human cells, mouse cells, rat cells, bovine cells, porcine cells, hamster cells, and monkey cells. In a particular embodiment, the cells that have been transfected with an expression vector that directs the expression of the melanocortin receptor are selected from the group consisting of: L cells L-M(TK$^-$) (ATCC CCL 1.3), L cells L-M (ATCC CCL 1.2), 293 cells (ATCC CRL 1573), Raji cells (ATCC CCL 86), CV-1 (ATCC CCL 70), COS-1 (ATCC CRL 1650), COS-7 (ATCC CRL 1651), CHO-K1 (ATCC CCL 61), 3T3 (ATCC CCL 92), NIH/3T3 (ATCC CRL 1658), HeLa (ATCC CCL 2), C127I (ATCC CRL 1616), BS-C-1 (ATCC CCL 26) and MRC-5 (ATCC CCL 171). In a particular embodiment, the cells are not *Xenopus* melanophore cells.

In a particular embodiment, the melanocyte stimulating hormone is selected from the group consisting of: α-melanocyte stimulating hormone, β-melanocyte stimulating hormone, and γ-melanocyte stimulating hormone.

In a particular embodiment, the ART polypeptide has an amino acid sequence selected from the group consisting of: SEQ.ID.NOs. 1-19 and 20.

In particular embodiments of the above-described method, the method is practiced *in vitro* and the conditions under which the method is practiced are conditions that are typically used in the art for

the study of protein-protein interactions: *e.g.*, physiological pH; salt conditions such as those represented by such commonly used buffers as PBS or in tissue culture media; a temperature of about 4°C to about 55°C.

In particular embodiments of the above-described method, the chosen concentration of the melanocyte stimulating hormone is from 0.05 nM to 2.0 nM, preferably from 0.1 nM to 1.0 nM, and more preferably from 0.2 nM to 0.5 nM.

In particular embodiments of the above-described method, the chosen concentration of the ART polypeptide is from 10^{-12} M to 10^{-7} M.

In particular embodiments of the above-described method, the method is practiced *in vitro* and the melanocyte stimulating hormone is labeled, *e.g.*, enzymatically, radioactively, or the like, and the amount of binding of the melanocyte stimulating hormone to the melanocortin receptor is measured by determining the amount of label bound to the cells containing the melanocortin receptor.

Steps (b), (c), and (d) of the above-described method can be modified in that, rather than exposing intact cells to the melanocyte stimulating hormone, the ART polypeptide, or the substance, membranes can be prepared from the cells and the membranes can be exposed to the melanocyte stimulating hormone, the ART polypeptide, or the substance. Such a modification utilizing membranes rather than intact cells in methods similar to that described above, although directed to the binding interactions of other ligands and receptors, is well known in the art and is described in, *e.g.*, Hess *et al*, 1992, Biochem. Biophys. Res. Comm. 184:260-268.

As a further modification of the above-described method, RNA encoding the melanocortin receptor can be prepared as, *e.g.*, by *in vitro* transcription using a plasmid containing nucleotide sequences encoding the melanocortin receptor under the control of a bacteriophage T7 promoter, and the RNA can be microinjected into *Xenopus* oocytes in order to cause the expression of the melanocortin receptor in the oocytes. These oocytes then take the place of the cells in the above described method.

Once a substance has been identified as an inhibitor of the binding of the the ART polypeptide to the melanocortin receptor, that substance can be tested to determine whether it is also an agonist of the

melanocortin receptor. Such testing would involve exposing cells that express the melanocortin receptor to the substance, in the absence of the melanocyte stimulating hormone and the ART protein or ART polypeptides, and determining whether the melanocortin receptor is thereby activated by the substance. In this way, an inhibitor of the effect of ART protein on MC3R or MC4R can be identified that has no, or little, MC3R or MC4R agonist activity, but that relieves the inhibition of MC3R or MC4R receptor activity produced by ART protein. In a similar manner, it can be determined whether the substance is an antagonist of the melanocortin receptor.

The present invention also includes a method for determining whether a substance is an inhibitor of the binding of an ART polypeptide to a melanocortin receptor where the method comprises:

(a) providing cells expressing a melanocortin receptor;

(b) exposing the cells to an ART polypeptide in the presence and in the absence of the substance under conditions such that if the substance were not present, the ART polypeptide would bind to the melanocortin receptor;

(c) measuring the amount of binding of the ART polypeptide to the melanocortin receptor in the presence and in the absence of the substance;

where a decrease in the amount of binding of the ART polypeptide to the melanocortin receptor in the presence as compared to the absence of the substance indicates that the substance is an inhibitor of the binding of the ART polypeptide to the melanocortin receptor.

In a particular embodiment, the cells expressing the melanocortin receptor are cells that naturally express the melanocortin receptor. In another embodiment, the cells expressing the melanocortin receptor do not naturally express the melanocortin receptor but have been transfected with an expression vector that directs the expression of the melanocortin receptor. Transfection is meant to include any method known in the art for the introduction of the the expression vector directing the expression of the melanocortin receptor into the cells. For example, transfection includes calcium phosphate or calcium chloride mediated transfection, lipofection, infection with a retroviral construct containing the melanocortin receptor, and electroporation.

In a particular embodiment, the melanocortin receptor is selected from the group consisting of: the melanocortin-3 receptor (MC3R) and the melanocortin-4 receptor (MC4R). In a particular embodiment of the above-described method, the melanocortin receptor is not a *Xenopus* melanocortin receptor.

The cells that have been transfected with an expression vector that directs the expression of the melanocortin receptor can be prokaryotic cells or eukaryotic cells. In a particular embodiment, the cells that have been transfected with an expression vector that directs the expression of the melanocortin receptor are selected from the group consisting of: yeast cells, mammalian cells, bacterial cells, and insect cells. In a particular embodiment, the cells that have been transfected with an expression vector that directs the expression of the melanocortin receptor are selected from the group consisting of: human cells, mouse cells, rat cells, bovine cells, porcine cells, hamster cells, and monkey cells. In a particular embodiment, the cells that have been transfected with an expression vector that directs the expression of the melanocortin receptor are selected from the group consisting of: L cells L-M(TK$^-$) (ATCC CCL 1.3), L cells L-M (ATCC CCL 1.2), 293 (ATCC CRL 1573), Raji (ATCC CCL 86), CV-1 (ATCC CCL 70), COS-1 (ATCC CRL 1650), COS-7 (ATCC CRL 1651), CHO-K1 (ATCC CCL 61), 3T3 (ATCC CCL 92), NIH/3T3 (ATCC CRL 1658), HeLa (ATCC CCL 2), C127I (ATCC CRL 1616), BS-C-1 (ATCC CCL 26) and MRC-5 (ATCC CCL 171). In a particular embodiment, the cells are not *Xenopus* melanophore cells.

In a particular embodiment, the ART polypeptide has an amino acid sequence selected from the group consisting of: SEQ.ID.NOs.1-19 and 20. In particular embodiments of the above-described method, the ART polypeptide is used in a concentration of from 10^{-12} M to 10^{-7} M.

In particular embodiments of the above-described method, the method is practiced *in vitro* and the conditions are conditions that are typically used in the art for the study of protein-protein interactions: *e.g.*, physiological pH; salt conditions such as those represented by such commonly used buffers as PBS or in tissue culture media; a temperature of about 4°C to about 55°C.

In particular embodiments of the above-described method, the method is practiced *in vitro* and the ART polypeptide is labeled, *e.g.*,

enzymatically, radioactively, or the like, and the amount of binding of the ART polypeptide to the melanocortin receptor is measured by determining the amount of label bound to the melanocortin receptor. The ART polypeptide will either be radioactively labeled by ^{32}P, ^{33}P, or ^{125}I (*e.g.*, for c-ART-a or c-ART-c), or non-radioactively labeled (*e.g.*, ART-AP, ART-luc, c-ART-AP or c-ART-luc). In the case of these latter ART polypeptides, the ART polypeptides can be detected by detecting the enzymatic activity of the alkaline phosphatase or luciferase moieties of the polypeptides.

Step (b) of the above-described method can be modified in that, rather than exposing the cells to an ART polypeptide in the presence and in the absence of the substance, membranes can be prepared from the cells and the membranes can be exposed to an ART polypeptide in the presence and in the absence of the substance. Such a modification utilizing membranes rather than cells in methods similar to that described above, although directed to the binding interactions of other ligands and receptors, is well known in the art and is described in, *e.g.*, Hess *et al*, 1992, Biochem. Biophys. Res. Comm. 184:260-268.

As a further modification of the above-described method, RNA encoding a melanocortin receptor can be prepared as, *e.g.*, by *in vitro* transcription using a plasmid containing nucleotide sequences encoding a melanocortin receptor under the control of a bacteriophage T7 promoter, and the RNA can be microinjected into *Xenopus* oocytes in order to cause the expression of the melanocortin receptor in the oocytes. These oocytes then take the place of the cells in the above described method.

Once a substance has been identified as an inhibitor of ART binding to a melanocortin receptor, that substance can be tested to determine whether it is also an agonist of the melanocortin receptor. Such testing would involve exposing cells that express the melanocortin receptor to the substance, in the absence of ART protein or ART polypeptides, and determining whether the melanocortin receptor is thereby activated by the substance. In this way, an inhibitor of ART protein binding to MC3R or MC4R may be identified that has no, or little, MC3R or MC4R agonist activity, but that relieves the inhibition of MC3R or MC4R receptor activity produced by ART protein.

The present invention also includes a method for determining whether a substance is an allosteric enhancer of the binding of an ART polypeptide to a melanocortin receptor where the method comprises:

(a) providing cells expressing a melanocortin receptor;

(b) exposing the cells to an ART polypeptide in the presence and in the absence of the substance under conditions such that if the substance were not present, the ART polypeptide would bind to the melanocortin receptor;

(c) measuring the amount of binding of the ART polypeptide to the melanocortin receptor in the presence and in the absence of the substance;

where an increase in the amount of binding of the ART polypeptide to the melanocortin receptor in the presence as compared to the absence of the substance indicates that the substance is an allosteric enhancer of the binding of the ART polypeptide to the melanocortin receptor.

In a particular embodiment, the cells expressing the melanocortin receptor are cells that naturally express the melanocortin receptor. In another embodiment, the cells expressing the melanocortin receptor do not naturally express the melanocortin receptor but have been transfected with an expression vector that directs the expression of the melanocortin receptor. Transfection is meant to include any method known in the art for the introduction of the the expression vector directing the expression of the melanocortin receptor into the cells. For example, transfection includes calcium phosphate or calcium chloride mediated transfection, lipofection, infection with a retroviral construct containing the melanocortin receptor, and electroporation.

In a particular embodiment, the melanocortin receptor is selected from the group consisting of: the melanocortin-3 receptor (MC3R) and the melanocortin-4 receptor (MC4R). In a particular embodiment of the above-described method, the melanocortin receptor is not a *Xenopus* melanocortin receptor.

The cells that have been transfected with an expression vector that directs the expression of the melanocortin receptor can be prokaryotic cells or eukaryotic cells. In a particular embodiment, the cells that have been transfected with an expression vector that directs

the expression of the melanocortin receptor are selected from the group consisting of: yeast cells, mammalian cells, bacterial cells, and insect cells. In a particular embodiment, the cells that have been transfected with an expression vector that directs the expression of the melanocortin receptor are selected from the group consisting of: human cells, mouse cells, rat cells, bovine cells, porcine cells, hamster cells, and monkey cells. In a particular embodiment, the cells that have been transfected with an expression vector that directs the expression of the melanocortin receptor are selected from the group consisting of: L cells L-M(TK$^-$) (ATCC CCL 1.3), L cells L-M (ATCC CCL 1.2), 293 (ATCC CRL 1573), Raji (ATCC CCL 86), CV-1 (ATCC CCL 70), COS-1 (ATCC CRL 1650), COS-7 (ATCC CRL 1651), CHO-K1 (ATCC CCL 61), 3T3 (ATCC CCL 92), NIH/3T3 (ATCC CRL 1658), HeLa (ATCC CCL 2), C127I (ATCC CRL 1616), BS-C-1 (ATCC CCL 26) and MRC-5 (ATCC CCL 171). In a particular embodiment, the cells are not *Xenopus* melanophore cells.

In a particular embodiment, the ART polypeptide has an amino acid sequence selected from the group consisting of: SEQ.ID.NOs.1-19 and 20. In particular embodiments of the above-described method, the ART polypeptide is used in a concentration of from 10^{-12} M to 10^{-7} M.

In particular embodiments of the above-described method, the method is practiced *in vitro* and the conditions are conditions that are typically used in the art for the study of protein-protein interactions: *e.g.*, physiological pH; salt conditions such as those represented by such commonly used buffers as PBS or in tissue culture media; a temperature of about 4°C to about 55°C.

In particular embodiments of the above-described method, the method is practiced *in vitro* and the ART polypeptide is labeled, *e.g.*, enzymatically, radioactively, or the like, and the amount of binding of the ART polypeptide to the melanocortin receptor is measured by determining the amount of label bound to the melanocortin receptor.

Step (b) of the above-described method can be modified in that, rather than exposing the cells to an ART polypeptide in the presence and in the absence of the substance, membranes can be prepared from the cells and the membranes can be exposed to an ART polypeptide in the presence and in the absence of the substance. Such a modification utilizing membranes rather than cells in methods similar

to that described above, although directed to the binding interactions of other ligands and receptors, is well known in the art and is described in, *e.g.*, Hess *et al*, 1992, Biochem. Biophys. Res. Comm. 184:260-268.

As a further modification of the above-described method, RNA encoding a melanocortin receptor can be prepared as, *e.g.*, by *in vitro* transcription using a plasmid containing nucleotide sequences encoding a melanocortin receptor under the control of a bacteriophage T7 promoter, and the RNA can be microinjected into *Xenopus* oocytes in order to cause the expression of the melanocortin receptor in the oocytes. These oocytes then take the place of the cells in the above described method.

Melanocortin receptors are G-protein coupled receptors that stimulate G_S, leading to the production of cAMP (Cone *et al.*, 1996, Recent Prog. Hormone Res. 51:287-318). Thus, the ART polypeptides of the present invention can be used in a method for determining whether a substance is a functional inhibitor of the antagonistic effect of an ART polypeptide on a melanocortin receptor where the method comprises:

(a) providing cells expressing a melanocortin receptor;

(b) exposing the cells to a melanocyte stimulating hormone, thereby activating the melanocortin receptor and leading to the production of cAMP mediated by the melanocortin receptor;

(c) exposing the cells to an ART polypeptide in the presence and in the absence of the substance under conditions such that if the substance were not present, the ART polypeptide would inhibit the production of cAMP mediated by the melanocortin receptor;

(d) measuring the amount of cAMP produced the presence and in the absence of the substance;

where an increase in the amount of cAMP produced in the presence as compared to the absence of the substance indicates that the substance is a functional inhibitor of the antagonistic effect of the ART polypeptide on the melanocortin receptor.

In a particular embodiment, the cells expressing the melanocortin receptor are cells that naturally express the melanocortin receptor. In another embodiment, the cells expressing the melanocortin receptor do not naturally express the melanocortin receptor but have been transfected with an expression vector that directs the expression of the melanocortin receptor. Transfection is meant to include any method

known in the art for the introduction of the the expression vector directing the expression of the melanocortin receptor into the cells. For example, transfection includes calcium phosphate or calcium chloride mediated transfection, lipofection, infection with a retroviral construct containing the melanocortin receptor, and electroporation.

In a particular embodiment, the melanocortin receptor is selected from the group consisting of: the melanocortin-3 receptor (MC3R) and the melanocortin-4 receptor (MC4R). In a particular embodiment of the above-described method, the melanocortin receptor is not a *Xenopus* melanocortin receptor.

The cells that have been transfected with an expression vector that directs the expression of the melanocortin receptor can be prokaryotic cells or eukaryotic cells. In a particular embodiment, the cells that have been transfected with an expression vector that directs the expression of the melanocortin receptor are selected from the group consisting of: yeast cells, mammalian cells, bacterial cells, and insect cells. In a particular embodiment, the cells that have been transfected with an expression vector that directs the expression of the melanocortin receptor are selected from the group consisting of: human cells, mouse cells, rat cells, bovine cells, porcine cells, hamster cells, and monkey cells. In a particular embodiment, the cells that have been transfected with an expression vector that directs the expression of the melanocortin receptor are selected from the group consisting of: L cells L-M(TK$^-$) (ATCC CCL 1.3), L cells L-M (ATCC CCL 1.2), 293 (ATCC CRL 1573), Raji (ATCC CCL 86), CV-1 (ATCC CCL 70), COS-1 (ATCC CRL 1650), COS-7 (ATCC CRL 1651), CHO-K1 (ATCC CCL 61), 3T3 (ATCC CCL 92), NIH/3T3 (ATCC CRL 1658), HeLa (ATCC CCL 2), C127I (ATCC CRL 1616), BS-C-1 (ATCC CCL 26) and MRC-5 (ATCC CCL 171). In a particular embodiment, the cells are not *Xenopus* melanophore cells.

In a particular embodiment, the melanocyte stimulating hormone is selected from the group consisting of: α-melanocyte stimulating hormone, β-melanocyte stimulating hormone, and γ-melanocyte stimulating hormone.

In a particular embodiment, the ART polypeptide has an amino acid sequence selected from the group consisting of: SEQ.ID.NOs.1-19 and 20.

In particular embodiments of the above-described method, the method is practiced *in vitro* and the conditions are conditions that are typically used in the art for the study of protein-protein interactions: *e.g.*, physiological pH; salt conditions such as those represented by such commonly used buffers as PBS or in tissue culture media; a temperature of about 4°C to about 55°C.

Once a substance has been identified as a functional inhibitor of the antagonistic effect of an ART polypeptide on a melanocortin receptor, that substance can be tested to determine whether it is also an agonist of the melanocortin receptor. Such testing would involve exposing cells that express the melanocortin receptor to the substance, in the absence of ART protein or ART polypeptides, and determining whether the melanocortin receptor is thereby activated by the substance. In this way, an inhibitor of ART protein binding to MC3R or MC4R may be developed that has no, or little, MC3R or MC4R agonist activity, but that relieves the inhibition of MC3R or MC4R receptor activity produced by ART protein. In a similar manner, it can be determined whether the substance is an antagonist of the melanocortin receptor.

The ART polypeptides of the present invention can also be used in a method of determining whether a substance is an inhibitor of the effect of an ART polypeptide that makes use of an assay utilizing a *Xenopus* melanophore cell line (see, *e.g.*, Quillan *et al.*, 1995, Proc. Natl. Acad. Sci. USA 92:2894; Potenza & Lerner, 1992, Pigment Cell Res. 5:372; Ollman *et al.*, 1997, Science 278:135-138). Such a method comprises:

(a) providing a *Xenopus* melanophore cell line;

(b) exposing the *Xenopus* melanophore cell line to a chosen concentration of a melanocyte stimulating hormone in the absence of the ART polypeptide and in the absence of the substance and measuring the amount of pigment dispersion to obtain a first value for pigment dispersion;

(c) exposing the *Xenopus* melanophore cell line to the chosen concentration of α-melanocyte stimulating hormone in the presence of the ART polypeptide and in the absence of the substance and measuring the amount of pigment dispersion to obtain a second value for pigment dispersion where the second value for pigment dispersion

indicates that less pigment has been dispersed as compared to the first value for pigment dispersion;

(d) exposing the *Xenopus* melanophore cell line to the chosen concentration of α-melanocyte stimulating hormone in the presence of the ART polypeptide and in the presence of the substance and measuring the amount of pigment dispersion to obtain a third value for pigment dispersion;

where if the third value for pigment dispersion indicates that more pigment has been dispersed as compared with the second value, then the substance is an inhibitor of the effect of the ART polypeptide.

In a particular embodiment, the melanocyte stimulating hormone is selected from the group consisting of: α-melanocyte stimulating hormone, β-melanocyte stimulating hormone, and γ-melanocyte stimulating hormone.

In a particular embodiment, the ART polypeptide has an amino acid sequence selected from the group consisting of: SEQ.ID.NOs.1-19 and 20.

In particular embodiments of the above-described method, the conditions are conditions that are typically used in the art for the study of protein-protein interactions: *e.g.*, physiological pH; salt conditions such as those represented by such commonly used buffers as PBS or in tissue culture media; a temperature of about 4°C to about 55°C.

Once a substance has been identified as an inhibitor of the effect of an ART polypeptide, that substance can be tested to determine whether it is also an agonist of the *Xenopus* melanocortin receptor. Such testing would involve exposing melanophore cells that express the Xenopus melanocortin receptor to the substance, in the absence of ART protein or ART polypeptides, and determining whether the melanocortin receptor is thereby activated by the substance. In this way, an inhibitor of ART protein binding to the *Xenopus* melanocortin receptor can be identified that may be used as a lead to develop ART binding inhibitors for human MC3R or MC4R that have no, or little, MC3R or MC4R agonist activity, but that relieve the inhibition of MC3R or MC4R receptor activity produced by ART protein. In a similar manner, it can be determined whether the substance is an antagonist of the melanocortin receptor.

The present invention includes a method of determining whether a substance is an inhibitor of the binding of an ART polypeptide to a melanocortin receptor comprising:

(a) providing cells expressing the melanocortin receptor;

(b) exposing the cells to a chosen concentration of the melanocyte stimulating hormone and a chosen concentration of the ART polypeptide in the presence and in the absence of the substance and measuring the amount of melanocyte stimulating hormone binding to the cells in the presence and in the absence of the substance;

where an increase in the amount of melanocyte stimulating hormone binding in the presence of the substance indicates that the substance is an inhibitor of the binding of an ART polypeptide to a melanocortin receptor.

In a particular embodiment, the cells expressing the melanocortin receptor are cells that naturally express the melanocortin receptor. In another embodiment, the cells expressing the melanocortin receptor do not naturally express the melanocortin receptor but have been transfected with an expression vector that directs the expression of the melanocortin receptor. Transfection is meant to include any method known in the art for the introduction of the the expression vector directing the expression of the melanocortin receptor into the cells. For example, transfection includes calcium phosphate or calcium chloride mediated transfection, lipofection, infection with a retroviral construct containing the melanocortin receptor, and electroporation.

In a particular embodiment, the melanocortin receptor is selected from the group consisting of: the melanocortin-3 receptor (MC3R) and the melanocortin-4 receptor (MC4R). In a particular embodiment of the above-described method, the melanocortin receptor is not a *Xenopus* melanocortin receptor.

The cells that have been transfected with an expression vector that directs the expression of the melanocortin receptor can be prokaryotic cells or eukaryotic cells. In a particular embodiment, the cells that have been transfected with an expression vector that directs the expression of the melanocortin receptor are selected from the group consisting of: yeast cells, mammalian cells, bacterial cells, and insect cells. In a particular embodiment, the cells that have been transfected with an expression vector that directs the expression of the melanocortin

receptor are selected from the group consisting of: human cells, mouse cells, rat cells, bovine cells, porcine cells, hamster cells, and monkey cells. In a particular embodiment, the cells that have been transfected with an expression vector that directs the expression of the melanocortin receptor are selected from the group consisting of: L cells L-M(TK$^-$) (ATCC CCL 1.3), L cells L-M (ATCC CCL 1.2), 293 cells (ATCC CRL 1573), Raji cells (ATCC CCL 86), CV-1 (ATCC CCL 70), COS-1 (ATCC CRL 1650), COS-7 (ATCC CRL 1651), CHO-K1 (ATCC CCL 61), 3T3 (ATCC CCL 92), NIH/3T3 (ATCC CRL 1658), HeLa (ATCC CCL 2), C127I (ATCC CRL 1616), BS-C-1 (ATCC CCL 26) and MRC-5 (ATCC CCL 171). In a particular embodiment, the cells are not *Xenopus* melanophore cells.

In a particular embodiment, the melanocyte stimulating hormone is selected from the group consisting of: α-melanocyte stimulating hormone, β-melanocyte stimulating hormone, and γ-melanocyte stimulating hormone.

In a particular embodiment, the ART polypeptide has an amino acid sequence selected from the group consisting of: SEQ.ID.NOs.1-19 and 20.

In particular embodiments of the above-described method, the method is practiced *in vitro* and the conditions under which the method is practiced are conditions that are typically used in the art for the study of protein-protein interactions: *e.g.*, physiological pH; salt conditions such as those represented by such commonly used buffers as PBS or in tissue culture media; a temperature of about 4°C to about 55°C.

In particular embodiments of the above-described method, the chosen concentration of the melanocyte stimulating hormone is from 0.05 nM to 2.0 nM, preferably from 0.1 nM to 1.0 nM, and more preferably from 0.2 nM to 0.5 nM.

In particular embodiments of the above-described method, the chosen concentration of the ART polypeptide is from 10^{-12} M to 10^{-7} M.

In particular embodiments of the above-described method, the method is practiced *in vitro* and the melanocyte stimulating hormone is labeled, *e.g.*, enzymatically, radioactively, or the like, and the amount of binding of the melanocyte stimulating hormone to the

melanocortin receptor is measured by determining the amount of label bound to the cells containing the melanocortin receptor.

Step (b) of the above-described method can be modified in that, rather than exposing intact cells to the melanocyte stimulating hormone, the ART polypeptide, or the substance, membranes can be prepared from the cells and the membranes can be exposed to the melanocyte stimulating hormone, the ART polypeptide, or the substance. Such a modification utilizing membranes rather than intact cells in methods similar to that described above, although directed to the binding interactions of other ligands and receptors, is well known in the art and is described in, *e.g.*, Hess *et al*, 1992, Biochem. Biophys. Res. Comm. 184:260-268.

As a further modification of the above-described method, RNA encoding the melanocortin receptor can be prepared as, *e.g.*, by *in vitro* transcription using a plasmid containing nucleotide sequences encoding the melanocortin receptor under the control of a bacteriophage T7 promoter, and the RNA can be microinjected into *Xenopus* oocytes in order to cause the expression of the melanocortin receptor in the oocytes. These oocytes then take the place of the cells in the above described method.

Once a substance has been identified as an inhibitor of the binding of the the ART polypeptide to the melanocortin receptor, that substance can be tested to determine whether it is also an agonist of the melanocortin receptor. Such testing would involve exposing cells that express the melanocortin receptor to the substance, in the absence of the melanocyte stimulating hormone and the ART protein or ART polypeptides, and determining whether the melanocortin receptor is thereby activated by the substance. In this way, an inhibitor of the effect of ART protein on MC3R or MC4R can be identified that has no, or little, MC3R or MC4R agonist activity, but that relieves the inhibition of MC3R or MC4R receptor activity produced by ART protein. In a similar manner, it can be determined whether the substance is an antagonist of the melanocortin receptor.

Compared to full-length ART protein, the ART polypeptides of the present invention are smaller, and therefore easier to produce and less likely to be degraded. With respect to such embodiments of the invention as, *e.g.*, c-ART-b, the non-ART protein amino acid sequences

added to the C-terminus of the ART sequences do not impair binding or functional activity, and allow ^{32}P or ^{33}P labeling without the need to label the ART sequence. Fusion polypeptides such as, *e.g.*, ART-AP or ART-luc, allow the use of non-radioactive methods to detect ART polypeptides in binding assays.

That the ART polypeptides of the present invention having amino acid sequences from non-ART proteins at their C-terminus are functional is surprising. The C-terminus of ART protein is homologous to the C-terminus of the agouti protein, both the ART protein and the agouti protein having a characteristic pattern of cysteine residues in this region. A similar pattern of cysteine residues has been found in certain ion channel blockers from spider and snail toxins. This pattern of cysteines has been proposed to result in the formation of specific disulfide bridges that constrain the toxins into a characteristic three-dimensional structure that is responsible for the toxins' biological activity (Kim *et al.*, 1995, J. Mol. Biol. 250:659-671; hereinafter "Kim"). While the extreme C-terminal amino acids of the toxin studied by Kim were not part of this three-dimensional structure, these extreme C-terminal amino acids were nevertheless "crucially important," since altering them resulted in a loss of activity. See page 665, right column of Kim: "These results suggest that the C-terminal segment of ω-AGA-IVA is crucially important for its blocking action on the P-type calcium channel expressed in rat cerebellar Purkinje cells." Thus, one would have expected that altering the C-terminus of the ART protein, *e.g.*, by linking it to sequences from a non-ART protein, would have resulted in an ART fusion polypeptide which would lack the activity of the full-length ART protein, or at least show substantially less activity. The present invention demonstrates that this is not so.

The following non-limiting examples are presented to better illustrate the invention.

EXAMPLE 1

Production of a construct expressing c-ART-b

The expression plasmid for c-ART-b was constructed by modifying the ART expression plasmid which was generated by inserting the ART cDNA into the EcoRI and BamHI sites of pcDNA3.1-Myc-His-A (Fong *et al.*, 1997, Biochem. Biophys. Res. Comm. 237:629-631; hereinafter "Fong"). The c-ART-b sequence differs from that of the recombinant ART as described by Fong in that residues 27-75 were deleted from ART. To accomplish that, a first PCR was carried out using the ART expression plasmid as template and two oligos (GGGCTCGGCGGTCCTGCAGGGCCAAGCCCATCTGGGC (SEQ.ID.NO.:21); and the T7 primer TAATACGACTCACTATAGGG (SEQ.ID.NO.:22)) to amplify the DNA fragment encoding ART residues 1-26 followed immediately by residues 76-81. A second PCR was carried out using the ART expression plasmid as template and two other oligos (CTGCAGGACCGCGAGCCC (SEQ.ID.NO.:23); and the pcDNA3.1A primer GTCGACGGCGCTATTCAG (SEQ.ID.NO.:24)) to amplify the DNA fragment encoding ART residues 76-132. A third PCR was then carried out using the first and the second PCR products as template and two oligos (the T7 primer and the pcDNA3.1A primer) to amplify the c-ART-b cDNA. The final PCR product was cleaved by the restriction enzymes EcoRI and BamHI, and ligated to the pcDNA3.1-Myc-His-A vector similarly cleaved by EcoRI and BamHI. The thrombin site sequence was based on the thrombin site in pET-34b (Novagen, Milwaukee, WI). The Myc epitope sequence and hexahistine sequence were contained within the pcDNA3.1-Myc-His-A vector (Invitrogen, Carlsbad, CA).

EXAMPLE 2

Expression of c-ART-b

COS-7 cells in T-175 flasks were transiently transfected with the c-ART-b expression plasmid (24 μg) by lipofectamine (Gibco), and grown in Opti-mem media (Gibco) supplemented with 1% fetal bovine

serum. Two days after transfection, culture media were collected, centrifuged to remove residual cells, concentrated about 100-fold in Centriprep-3 (Amicon) and stored at 4 °C in the presence of 2.5 mM EGTA, 4 mg/ml leupeptin, and 0.01 mM phosphoramidon. After determination of the concentration of c-ART-b, NaN_3 was added to 0.02%. Determination of the concentration of c-ART-b, which contains the Myc sequence, was based on an ELISA standard curve. Briefly, the microtiter plate was coated with 0.2 µg of a Myc peptide (human c-Myc 408-439) overnight, washed, blocked, and followed by incubation with anti-Myc mAb-HRP conjugates (Invitrogen) in the presence of varying concentrations of the free Myc peptide for 2 hours. The bound mAb-HRP was detected using a colorimetric substrate tetramethylbenzidine (BioRad). For c-ART-b concentration determination, a c-ART-b sample replaced the free Myc peptide.

EXAMPLE 3

Binding of c-ART-b to MC3R and MC4R

Binding assays were done in the same manner as described in Fong *et al.*, 1997, Biochem. Biophys. Res. Comm. 237:629-631. Binding assays were carried out using membranes prepared from L cells or CHO cells stably expressing human MC3R, MC4R or MC5R. The binding assay mixture contained 0.2 nM of ^{125}I-[Tyr^2][Nle^4, D-Phe^7] α-melanocyte stimulating hormone (^{125}I -NDP-α-MSH), varying concentrations of c-ART-b or full-length ART protein, and an appropriate amount of membranes so that the total bound radioligand was less than 10% of the added radioligand. The above mixture in binding buffer (50 mM Tris, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 5 mM KCl, pH 7.2) was incubated at room temperature for 2 hours, followed by filtration through GFC paper. The bound ligand was quantitated in a γ counter. IC_{50} values were calculated as previously described (Fong *et al.*, 1996, Mol. Pharmacol. 50:1605-1611). The results are shown in Figure 2. From Figure 2 it can be seen that c-ART-b inhibits the binding of ^{125}I-NDP-α-MSH to MC3R.

A similar experiment was done to determine whether c-ART-b inhibits the binding of ^{125}I-labeled NDP-α-MSH to MC4R. The

results are shown in Figure 3. From Figure 3 it can be seen that c-ART-b inhibits the binding of ^{125}I-NDP-α-MSH to hMC4R.

Similar experiments were performed with full-length human ART protein. Similar experiments were also performed with full-length ART protein and with c-ART-b for the melanocortin-5 receptor (MC5R). From these experiments, from the results shown in Figures 2 and 3, and from similar experiments, the following IC_{50} values for the inhibition of ^{125}I-labeled NDP-α-MSH to MC3R, MC4R, and MC5R by full-length ART protein and by c-ART-b can be determined.

Table 1

	hMC3R	hMC4R	hMC5R
full length ART	1.0Ò0.4 (4)	0.5Ò0.1 (3)	>40
c-ART-b	1.9Ò1.0 (2)	1.4Ò0.1 (2)	not done

The IC_{50} values shown in Table 1 are given in nM. The numbers in parentheses represent the number of experiments run. The results shown in Table 1 indicate that, surprisingly, c-ART-b, although missing a significant amount of sequence from the N-terminus of the ART protein, is essentially functionally equivalent to full length ART protein. In addition, c-ART-b is functional despite having a significant amount of non-ART sequences at its C-terminus (a thrombin site, a myc epitope, and a hexahistidine tag).

EXAMPLE 4

Functional assay for the binding of c-ART-b to MC3R and MC4R

The ability of c-ART-b to inhibit the production of cAMP by α-melanocyte stimulating hormone acting through MC3R or MC4R can be demonstrated by preincubating L Cells stably expressing human MC3R or MC4R with c-ART-b for 10 minutes, followed by incubation with 20 nM α-melanocyte stimulating hormone for 45 minutes. The incubation buffer also contains Earle's balanced salt solution, 10 mM

HEPES, 5 mM $MgCl_2$, 1 mg/ml BSA and 0.5 mM IBMX. -Following the incubation, cells are lysed by boiling for 4 minutes. Intracellular cAMP concentration is measured by RIA (Huang *et al.*, 1997, J. Receptor Signal Transduc. Res. 17:599-607) using anti-cAMP antibody and ^{125}I-cAMP as modified in the scintillation proximity assay format (Amersham).

The present invention is not to be limited in scope by the specific embodiments described herein. Indeed, various modifications of the invention in addition to those described herein will become apparent to those skilled in the art from the foregoing description. Such modifications are intended to fall within the scope of the appended claims.

Various publications are cited herein, the disclosures of which are incorporated by reference in their entireties.

WHAT IS CLAIMED:

1. A fusion protein having an amino acid sequence from the ART protein fused at its carboxy terminus to one or more amino acid sequences not derived from the ART protein, where the amino acid sequence from the ART protein is selected from the group consisting of: SEQ.ID.NOs.:6-8 and 9.

2. The fusion protein of claim 1 having an amino acid sequence selected from the group consisting of: SEQ.ID.NOs.:1-5, 10-19, and 20.

3. The fusion protein of claim 2 having the amino acid sequence of SEQ.ID.NO.:2.

4. An ART polypeptide having an amino acid sequence selected from the group consisting of: SEQ.ID.NOs.:6-8 and 9.

5. A DNA sequence encoding the fusion protein of claim 1.

6. A method of determining whether a substance is an inhibitor of the binding of an ART polypeptide to a melanocortin receptor where the method comprises:

(a) providing cells expressing the melanocortin receptor;

(b) exposing the cells to a chosen concentration of the melanocyte stimulating hormone in the absence of the ART polypeptide and in the absence of the substance and measuring the amount of melanocyte stimulating hormone binding to the cells to obtain a first value for melanocyte stimulating hormone binding;

(c) exposing the cells to the chosen concentration of melanocyte stimulating hormone in the presence of a chosen concentration of the ART polypeptide and in the absence of the substance and measuring the amount of melanocyte stimulating hormone binding to obtain a second value for melanocyte stimulating hormone binding where the second value for melanocyte stimulating hormone binding indicates that less melanocyte stimulating hormone binding has

occurred as compared to the first value for melanocyte stimulating hormone binding;

(d) exposing the cells to the chosen concentration of melanocyte stimulating hormone in the presence of the chosen concentration of ART polypeptide and in the presence of the substance and measuring the amount of melanocyte stimulating hormone binding to obtain a third value for melanocyte stimulating hormone binding;

where, if the third value for melanocyte stimulating hormone binding is greater than the second value, then the substance is an inhibitor of the binding of the ART polypeptide to the melanocortin receptor;

where the ART polypeptide has an amino acid sequence selected from the group consisting of: SEQ.ID.NOs.:1-19 and 20.

7. The method of claim 6 where the melanocortin receptor is selected from the group consisting of: the melanocortin-3 receptor (MC3R) and the melanocortin-4 receptor (MC4R).

8. A method for determining whether a substance is an inhibitor of the binding of an ART polypeptide to a melanocortin receptor where the method comprises:

(a) providing cells expressing a melanocortin receptor;

(b) exposing the cells to an ART polypeptide in the presence and in the absence of the substance under conditions such that if the substance were not present, the ART polypeptide would bind to the melanocortin receptor;

(c) measuring the amount of binding of the ART polypeptide to the melanocortin receptor in the presence and in the absence of the substance;

where a decrease in the amount of binding of the ART polypeptide to the melanocortin receptor in the presence as compared to the absence of the substance indicates that the substance is an inhibitor of the binding of the ART polypeptide to the melanocortin receptor;

where the ART polypeptide has an amino acid sequence selected from the group consisting of: SEQ.ID.NOs.:1-19 and 20.

9. The method of claim 8 where the melanocortin receptor is selected from the group consisting of: the melanocortin-3 receptor (MC3R) and the melanocortin-4 receptor (MC4R).

10. A method for determining whether a substance is an allosteric enhancer of the binding of an ART polypeptide to a melanocortin receptor where the method comprises:

(a) providing cells expressing a melanocortin receptor;

(b) exposing the cells to an ART polypeptide in the presence and in the absence of the substance under conditions such that if the substance were not present, the ART polypeptide would bind to the melanocortin receptor;

(c) measuring the amount of binding of the ART polypeptide to the melanocortin receptor in the presence and in the absence of the substance;

where an increase in the amount of binding of the ART polypeptide to the melanocortin receptor in the presence as compared to the absence of the substance indicates that the substance is an allosteric enhancer of the binding of the ART polypeptide to the melanocortin receptor;

where the ART polypeptide has an amino acid sequence selected from the group consisting of: SEQ.ID.NOs.:1-19 and 20.

11. The method of claim 10 where the melanocortin receptor is selected from the group consisting of: the melanocortin-3 receptor (MC3R) and the melanocortin-4 receptor (MC4R).

12. A method for determining whether a substance is a functional inhibitor of the antagonistic effect of an ART polypeptide on a melanocortin receptor where the method comprises:

(a) providing cells expressing a melanocortin receptor;

(b) exposing the cells to a melanocyte stimulating hormone selected from the group consisting of: α-melanocyte stimulating hormone, β-melanocyte stimulating hormone, and γ-melanocyte stimulating hormone, in order to activate the melanocortin receptor, leading to the production of cAMP;

(c) exposing the cells to an ART polypeptide in the presence and in the absence of the substance under conditions such that if the substance were not present, the ART polypeptide would inhibit the production of cAMP mediated by the melanocortin receptor;

(d) measuring the amount of cAMP produced the presence and in the absence of the substance;

where an increase in the amount of cAMP produced in the presence as compared to the absence of the substance indicates that the substance is a functional inhibitor of the antagonistic effect of the ART polypeptide on the melanocortin receptor;

where the ART polypeptide has an amino acid sequence selected from the group consisting of: SEQ.ID.NOs.:1-19 and 20.

13. The method of claim 12 where the melanocortin receptor is selected from the group consisting of: the melanocortin-3 receptor (MC3R) and the melanocortin-4 receptor (MC4R).

14. A method of determining whether a substance is an inhibitor of the effect of an ART polypeptide comprising:

(a) providing a *Xenopus* melanophore cell line;

(b) exposing the *Xenopus* melanophore cell line to a chosen concentration of α-melanocyte stimulating hormone in the absence of the ART polypeptide and in the absence of the substance and measuring the amount of pigment dispersion to obtain a first value for pigment dispersion;

(c) exposing the *Xenopus* melanophore cell line to the chosen concentration of α-melanocyte stimulating hormone in the presence of the ART polypeptide and in the absence of the substance and measuring the amount of pigment dispersion to obtain a second value for pigment dispersion where the second value for pigment dispersion indicates that less pigment has been dispersed as compared to the first value for pigment dispersion;

(d) exposing the *Xenopus* melanophore cell line to the chosen concentration of α-melanocyte stimulating hormone in the presence of the ART polypeptide and in the presence of the substance and measuring the amount of pigment dispersion to obtain a third value for pigment dispersion;

where if the third value for pigment dispersion indicates that more pigment has been dispersed as compared with the second value, then the substance is an inhibitor of the effect of the ART polypeptide;

where the ART polypeptide has an amino acid sequence selected from the group consisting of: SEQ.ID.NOs.:1-19 and 20.

15. A method of determining whether a substance is an inhibitor of the binding of an ART polypeptide to a melanocortin receptor comprising:

(a) providing cells expressing the melanocortin receptor;

(b) exposing the cells to a chosen concentration of the melanocyte stimulating hormone and a chosen concentration of the ART polypeptide in the presence and in the absence of the substance and measuring the amount of melanocyte stimulating hormone binding to the cells in the presence and in the absence of the substance;

where an increase in the amount of melanocyte stimulating hormone binding in the presence of the substance indicates that the substance is an inhibitor of the binding of an ART polypeptide to a melanocortin receptor;

where the ART polypeptide has an amino acid sequence selected from the group consisting of: SEQ.ID.NOs.:1-19 and 20.

16. The method of claim 15 where the melanocortin receptor is selected from the group consisting of: the melanocortin-3 receptor (MC3R) and the melanocortin-4 receptor (MC4R).

FIGURE 1

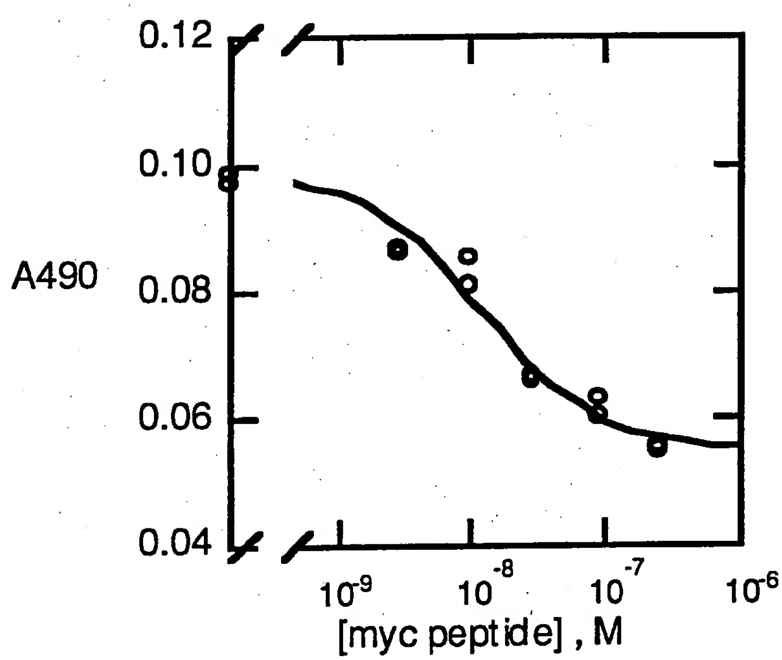
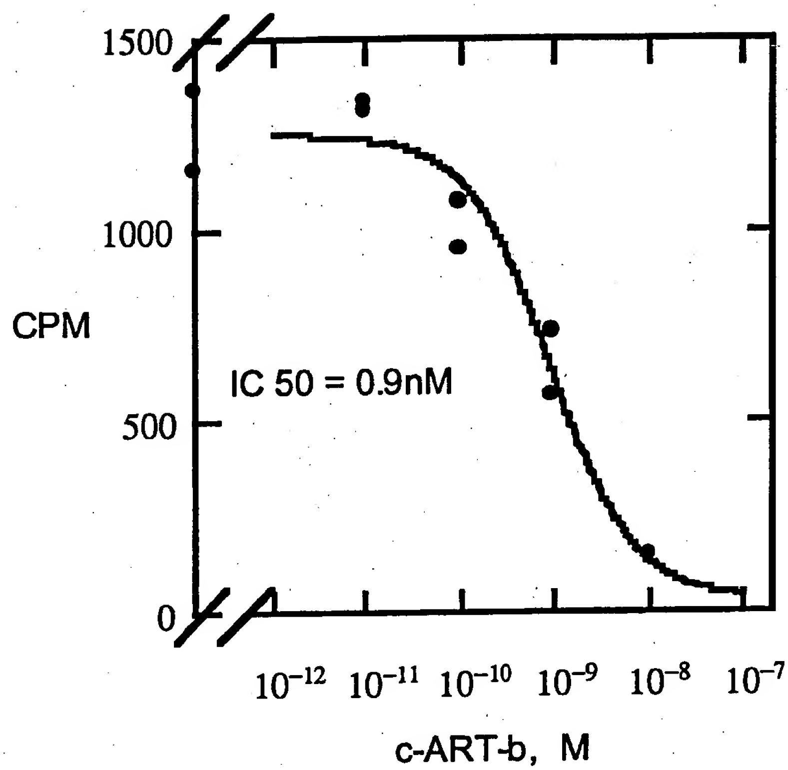
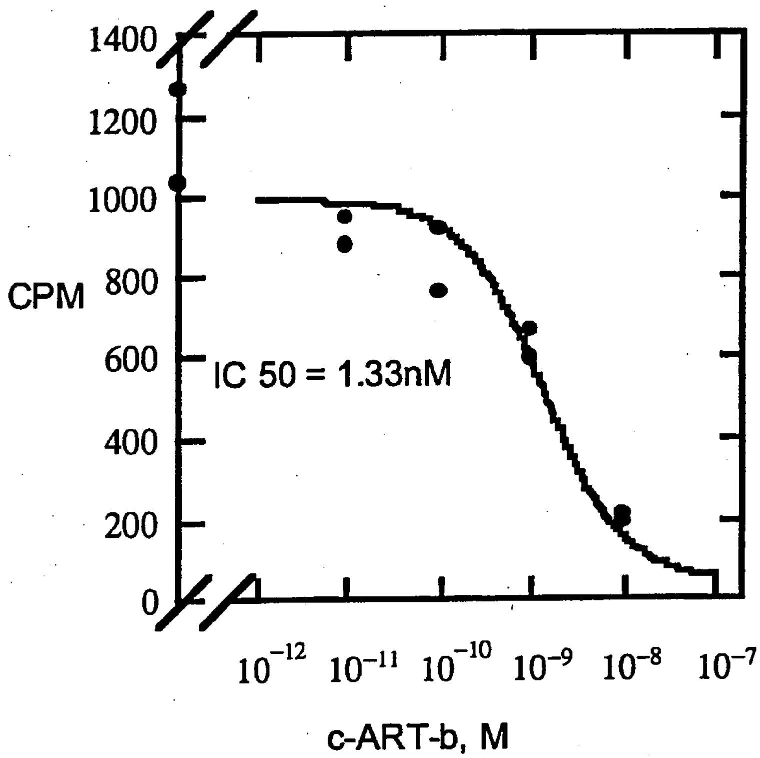


FIGURE 2


1500
1000
500
0
CPM
IC 50 = 0.9nM
10^{-12} 10^{-11} 10^{-10} 10^{-9} 10^{-8} 10^{-7}
c-ART-b, M

CPM

FIGURE 3


1400
1200
1000
800
600
400
200
0
CPM
IC 50 = 1.33nM
10^{-12} 10^{-11} 10^{-10} 10^{-9} 10^{-8} 10^{-7}
c-ART-b, M

CPM

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US98/26457

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :G01N 33/53, 33/566; C07K 14/435, 19/00; C12N 15/12
US CL :530/300, 350; 536/23.4; 436/501
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 530/300, 350; 536/23.4; 436/501

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS; DIALOG - Biotech Files; GenEMBL sequence databases

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	KWON, H. Y. et al. Molecular structure and chromosomal mapping of the human homolog of the agouti gene. Proceedings of the National Academy of Sciences, USA. October 1994, Vol. 91, pages 9760-9764, see entire document.	1-16
A	STARK, M. J. R. et al. The killer toxin of Kluyveromyces lactis: characterization of the toxin subunits and identification of the genes which encode them. The EMBO Journal. 1986, Vol. 5, No. 3, pages 1995-2002, see entire document.	1-16

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

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"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

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"&" document member of the same patent family

Date of the actual completion of the international search

08 APRIL 1999

Date of mailing of the international search report

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US98/26457

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	SHUTTER, J. R. et al. Hypothalamic expression of ART, a novel gene related to agouti, is up-regulated in obese and diabetic mutant mice. Genes and Development. 1997, Vol. 11, pages 593-602, see entire document.	1-16
Y,P	US 5,766,877 (STARK et al) 16 June 1998, columns 1-36, see entire document.	1-16

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(54) Title: MELANOCORTIN RECEPTOR LIGANDS AND METHODS OF USING SAME

(57) Abstract

The invention provides ligands for melanocortin receptors. For example, the invention provides the melanocortin receptor peptide ligand Ac–Nle–Gln–His–(*p*(I)–D–Phe)–Arg–(D–Trp)–Gly–NH_2, where the iodo group is unlabeled or radioactively labeled. The invention additionally provides methods of assaying for melanocortin receptors in a cell or tissue such as brain. The invention also relates to pharmaceutical compositions comprising a pharmaceutically acceptable carrier and a melanocortin receptor ligand and to methods of administering the pharmaceutical composition to a subject. The invention further provides tetrapeptide ligands for melanocortin receptors and methods of altering melanocortin receptor activity.

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MELANOCORTIN RECEPTOR LIGANDS AND METHODS OF USING SAME

BACKGROUND OF THE INVENTION

FIELD OF THE INVENTION

The present invention relates generally to the fields of peptide chemistry and molecular pathology and more specifically to novel melanocortin receptor ligands.

BACKGROUND INFORMATION

The melanocortin (MC) receptors are a group of cell surface proteins that mediate a variety of physiological effects, including regulation of adrenal gland function such as production of the glucocorticoid cortisol and aldosterone; control of melanocyte growth and pigment production; thermoregulation; immunomodulation; and analgesia. Five distinct MC receptors have been cloned and are expressed in a variety of tissues, including melanocytes, adrenal cortex, brain, gut, placenta, skeletal muscle, lung, spleen, thymus, bone marrow, pituitary, gonads and adipose tissue (Tatro, Neuroimmunomodulation 3:259-284 (1996)). Three MC receptors, MC1, MC3 and MC4, are expressed in brain tissue (Xia et al., Neuroreport 6:2193-2196 (1995)).

A variety of ligands termed melanocortins function as agonists that stimulate the activity of MC receptors. The melanocortins include melanocyte-stimulating hormones (MSH) such as α-MSH, β-MSH and γ-MSH, as well as adrenocorticotropic hormone (ACTH). Individual ligands can bind to multiple

MC receptors with differing relative affinities. The variety of ligands and MC receptors with differential tissue-specific expression likely provides the molecular basis for the diverse physiological effects of melanocortins and MC receptors.

A particularly potent MC receptor ligand is an α-MSH analogue, NDP. NDP has been used extensively to characterize MC receptors because it is chemically and enzymatically stable and binds with high affinity to all identified MC receptors. Despite the availability of NDP, no binding assay has been reported for the detection of MC receptors in brain tissue even though MC receptor messenger RNA is expressed in brain. Detection of MC receptors in brain is of particular interest since brain MC receptors mediate some of the physiological effects of melanocortins, including the antipyretic effect observed with experimentally induced fever.

Due to the varied physiological activities of MC receptors, high affinity ligands of MC receptors would be valuable to analyze the presence of MC receptors in particular cells or tissues. In addition, high affinity ligands of MC receptors could be used to exploit the varied physiological responses of MC receptors by functioning as potential therapeutic agents or as lead compounds for the development of therapeutic agents.

Thus, there exists a need for ligands that bind to MC receptors with high affinity and methods for detecting the presence of MC receptors in a cell or tissue such as brain. The present invention satisfies this need and provides related advantages as well.

SUMMARY OF THE INVENTION

The invention provides ligands for MC receptors. For example, the invention provides the MC receptor peptide ligand Ac-Nle-Gln-His-(*p*(I)-D-Phe)-Arg-(D-Trp)-Gly-NH_2, where the iodo group is unlabeled or radioactively labeled. The invention additionally provides methods of assaying for MC receptors in a cell or tissue such as brain. The invention also relates to pharmaceutical compositions comprising a pharmaceutically acceptable carrier and a melanocortin receptor ligand and to methods of administering the pharmaceutical composition to a subject. The invention further provides tetrapeptide ligands for MC receptors and methods of altering MC receptor activity.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows a saturation binding isotherm for ^{125}I-HP 467.

Figure 2 shows the association rate for ^{125}I-HP 467.

Figure 3 shows the dissociation rate for ^{125}I-HP 467.

Figure 4 shows a competition curve of unlabeled HP 467 for ^{125}I-HP 467.

Figure 5 shows an HP 467 saturation binding curve of mouse L cells expressing MC1.

Figure 6 shows an HP 467 saturation binding curve of mouse L cells expressing MC3.

Figure 7 shows an HP 467 saturation binding curve of mouse L cells expressing MC4.

Figure 8 shows a saturation binding isotherm for HP 467 on HEK 293 cells expressing MC1.

Figure 9 shows a saturation binding isotherm for HP 467 on HEK 293 cells expressing MC3.

Figure 10 shows a saturation binding isotherm for HP 467 on HEK 293 cells expressing MC4.

Figure 11 shows the percent bound for all library mixtures of a tetrapeptide scanning combinatorial library.

DETAILED DESCRIPTION OF THE INVENTION

The invention provides ligands for MC receptors and methods for detecting the presence of MC receptors in a cell or tissue. For example, the invention provides the MC receptor peptide ligand HP 467, having the amino acid sequence Ac-Nle-Gln-His-(*p*(I)-D-Phe)-Arg-(D-Trp)-Gly-NH_2 (SEQ ID NO:1). HP 467 is a *para*-iodinated form of HP 228 (see Table I), wherein the iodo group can be a stable nuclide such as ^{127}I or an unstable nuclide, for example, radioactive ^{125}I or ^{131}I. HP 228 is a heptapeptide analogue of NDP (see Table I) and, like NDP, is both more potent and more stable than α-MSH. Table I shows various MC receptor ligands.

Table I. Melanocortin Receptor Ligands

αMSH:	$Ac-SYSMEHFRWGKPV-NH_2$	(SEQ ID NO:2)
NDP:	$Ac-SYS(Nle)EHfRWGKPV-NH_2$	(SEQ ID NO:3)
γ1-MSH:	YVMGHFRWDRF-OH	(SEQ ID NO:4)
γ2-MSH:	$H_2N-YVMGHFRWDRFG-OH$	(SEQ ID NO:5)
γ3-MSH:	$H_2N-YVMGHFRWDRFGRRNGSSSSGVGGAAQ-OH$	(SEQ ID NO:6)
$ACTH_{(4-10)}$:	MEHFRWG-OH	(SEQ ID NO:7)
β-MSH:	$H_2N-AEKKDEGPYRMEHFRWGSPPKE-OH$	(SEQ ID NO:8)
HP 228:	$Ac-(Nle)QHfRwG-NH_2$	(SEQ ID NO:9)

Amino acids provided throughout the application are identified by their well known one letter or three letter codes and as being in the D- or L-configuration by designations "D" or "L," respectively or, alternatively, using lower case letters to designate amino acids in the D-configuration. Where not specified, an amino acid can be in the D- or L- configuration but is more likely in the L-form.

The structural relationship between HP 467 and various MC receptor ligands suggested that HP 467 could function as an MC receptor ligand. As disclosed herein, HP 467 exhibits high affinity binding to MC receptors in rat brain homogenates and in cell lines transfected with various MC receptors (see Examples III and IV).

The invention also provides MC receptor tetrapeptide ligands. A combinatorial library was prepared by the positional scanning format (U.S. Patent No. 5,556,762, issued September 17, 1996, which is incorporated herein by reference) and screened to find

smaller ligands that bind to MC receptors. The present invention thus further provides tetrapeptide ligands that bind to MC receptors. Such tetrapeptides have the structure A1-B2-C3-D4, where "A1" is αFmLys, L-hmP, His, L-Nal, Arg, D-Arg, ε-Lys, Lys, D-pyrala, D-Lys, D-His, D-Ala, Thiopro, L-isoN or 3-2Met; "B2" is Arg, D-Thi, *p*Cl-f, D-Phe, Arg, α-Orn, *p*F-F, D-His, D-Lys, ε-Lys, δ-Orn, Thiopro, t4-benz, L-hmP or D-Cit; "C3" is Arg, L-Cha, D-Ile, D-Arg, *p*Cl-F, D-Lys, α-Orn, *p*Cl-f, D-Ser, L-hmP, L-pyrala, D-His, Npecot, εAca, D-Cit or Thiopro; and "D4" is D-Nal, D-Arg, D-His, ε-Lys, Lys, D-Lys or D-Glu. Abbreviations of amino acid derivatives used throughout the application are shown in Table II.

As disclosed herein, a positional scanning combinatorial library was constructed to contain 91^4 (68,574,960) tetrapeptides having the general structure A1-B2-C3-D4. In positional scanning libraries, a defined amino acid is determined for a given position and is "walked" through the length of the peptide, resulting in the defined amino acid appearing in positions A1, B2, C3 and D4. Mixtures active at each of the four positions can be identified in a single screen. The following peptides were synthesized: Ac-OXXX-NH_2; Ac-XOXX-NH_2; Ac-XXOX-NH_2; and Ac-XXXO-NH_2; where "O" is a defined single amino acid and "X" represents a mixture of 91 L-, D- and amino acid derivatives such that each peptide is represented as 91 mixtures each containing 753,571 peptides.

Table II. Abbreviations of Amino Acid Derivatives

Abbreviation	Full Name
e-Aca	e-aminocaproic acid
*t*4-benz	*trans*-4-($NHCH_2$)cyclohexyl-COOH
Boc	t-butoxycarbonyl
Cbz	benzyloxycarbonyl
Cha	cyclohexylalanine
Cit	citrulline
Fmoc	fluorenylmethoxycarbonyl
αFmLys	lysine (α-Fmoc)
hmP	homoproline
isoN	isoasparagine
e-Lys	αN-Cbz-lysine (Boc)
3-2Met	3-amino-2-methyl-propionic acid
Nal	naphthylalanine
Npecot	nipecotic acid
α-Orn	ornithine (Cbz)
δ-Orn	αN-Cbz-ornithine (Boc)
*p*Cl-F	L-4-chlorophenylalanine
*p*Cl-f	D-4-chlorophenylalanine
*p*F-F	L-4-fluorophenylalanine
pyrala	(3-pyridyl)alanine
Thi	(2-thienyl)alanine
Thiopro	thioproline

Using a positional scanning combinatorial library, a number of high affinity tetrapeptide MC receptor ligands were identified (see Example X). Particularly active peptides are provided herein having the structure A1-B2-C3-D4, where "A1" is αFmLys or His; "B2" is Arg, D-Thi, or *p*Cl-f; "C3" is Arg, L-Cha, or

D-Ile; and "D4" is D-Nal or D-Arg (see Table V in Example X). Based on results with these peptides, additional peptides were synthesized with the following amino acids in specific positions in the tetrapeptide: αFmLys and His at position "A1"; Arg, D-Thi and *p*Cl-f at position "B2"; Arg, L-Cha and Ile at position "C3"; and D-Nal and D-Arg at position "D4". Synthesis of peptides containing these amino acids resulted in 36 individual tetrapeptides that were tested for activity. Several peptides were found to have high affinity for MC receptors (see Table VI in Example X).

The invention also provides the peptides His-(*p*Cl-f)-Arg-(D-Nal) (SEQ ID NO:10); His-(*p*Cl-f)-(L-Cha)-(D-Arg) (SEQ ID NO:11); (αFmLys)-(*p*Cl-f)-Arg-(Nal) (SEQ ID NO:12); (αFmLys)-Arg-(L-Cha)-(Nal) (SEQ ID NO:13); (αFmLys)-Arg-(L-Cha)-(D-Arg) (SEQ ID NO:14); (αFmLys)-(D-Thi)-Arg-(Nal) (SEQ ID NO:15); (αFmLys)-Arg-Arg-(Nal) (SEQ ID NO:16); and His-(*p*Cl-f)-Arg-(D-Nal) (SEQ ID NO:17). The amino terminus for any of the tetrapeptides disclosed herein can be modified by acetylation and the carboxy terminus can be modified by amidation. As disclosed herein, the peptide Ac-His-(*p*Cl-f)-Arg-(D-Nal)-NH_2 (SEQ ID NO:18) is a high affinity MC receptor ligand having an IC_{50}, the inhibitory concentration at which 50% of binding is inhibited, of 18 nM.

MC receptor ligands such as the peptides disclosed herein can be synthesized using a modification of the solid phase peptide synthesis method of Merrifield (J. Am. Chem. Soc. 85:2149 (1964), which is incorporated herein by reference) or can be synthesized using standard solution methods well known in the art (see, for example,

Bodanszky, M., Principles of Peptide Synthesis (Springer-Verlag, 1984), which is incorporated herein by reference). Peptides prepared by the method of Merrifield can be synthesized using an automated peptide synthesizer such as the Applied Biosystems 431A-01 Peptide Synthesizer (Mountain View, CA) or using the manual peptide synthesis technique described by Houghten, Proc. Natl. Acad. Sci., *USA* 82:5131 (1985), which is incorporated herein by reference. For example, HP 467 was synthesized as described in Example I.

Peptides can be synthesized using amino acids or amino acid analogs, the active groups of which are protected as required using, for example, a t-butyldicarbonate (t-BOC) group or a fluorenylmethoxy carbonyl (FMOC) group. Amino acids and amino acid analogs can be purchased commercially (Sigma Chemical Co.; Advanced Chemtec) or synthesized using methods known in the art. Peptides synthesized using the solid phase method can be attached to resins including 4-methylbenzhydrylamine (MBHA), 4-(oxymethyl)-phenylacetamido methyl and 4-(hydroxymethyl)phenoxymethyl-copoly(styrene-1% divinylbenzene) (Wang resin), all of which are commercially available, or to p-nitrobenzophenone oxime polymer (oxime resin), which can be synthesized as described by De Grado and Kaiser, J. Org. Chem. 47:3258 (1982), which is incorporated herein by reference.

The choice of amino acids or amino acid derivatives incorporated into the peptide will depend, in part, on the specific physical, chemical or biological characteristics required of the MC receptor peptide ligand. Such characteristics are determined, in part, by the route by which the MC receptor ligand will be

administered or the location in a subject to which the MC receptor ligand will be directed.

Selective modification of the reactive groups in a peptide also can impart desirable characteristics to an MC receptor ligand. Peptides can be manipulated while still attached to the resin to obtain N-terminal modified compounds such as an acetylated peptide or can be removed from the resin using hydrogen fluoride or an equivalent cleaving reagent, then modified. Compounds synthesized containing the C-terminal carboxy group (Wang resin) can be modified after cleavage from the resin or, in some cases, prior to solution phase synthesis. Methods for modifying the N-terminus or C-terminus of a peptide are well known in the art and include, for example, methods for acetylation of the N-terminus or methods for amidation of the C-terminus. Similarly, methods for modifying side chains of the amino acids or amino acid analogs are well known to those skilled in the art of peptide synthesis. The choice of modifications made to the reactive groups present on the peptide will be determined by the characteristics that are desired in the peptide.

A cyclic peptide also can be an effective MC receptor ligand. A cyclic peptide can be obtained by inducing the formation of a covalent bond between, for example, the amino group at the N-terminus of the peptide and the carboxyl group at the C-terminus. Alternatively, a cyclic peptide can be obtained by forming a covalent bond between a terminal reactive group and a reactive amino acid side chain or between two reactive amino acid side chains. The choice of a particular cyclic peptide is determined by the reactive groups present on the peptide as well as the desired characteristic of the

peptide. For example, a cyclic peptide can provide an MC receptor ligand with increased stability *in vivo*.

A newly synthesized peptide can be purified using a method such as reverse phase high performance liquid chromatography (RP-HPLC) as described in United States Patent No. 5,420,109, issued May 30, 1995, which is incorporated herein by reference. Alternatively, other methods of separation based on the size or charge of the peptide can be used for peptide purification. Furthermore, the purified peptide can be characterized using these and other well known methods such as amino acid analysis and mass spectrometry (see U.S. Patent No. 5,420,109).

The invention also relates to pharmaceutical compositions comprising an MC receptor ligand such as HP 467 and a pharmaceutically acceptable carrier. Pharmaceutically acceptable carriers are well known in the art and include aqueous solutions such as physiologically buffered saline or other solvents or vehicles such as glycols, glycerol, oils such as olive oil or injectable organic esters.

A pharmaceutically acceptable carrier can contain physiologically acceptable compounds that act, for example, to stabilize the MC receptor ligand or increase the absorption of the agent. Such physiologically acceptable compounds include, for example, carbohydrates, such as glucose, sucrose or dextrans, antioxidants, such as ascorbic acid or glutathione, chelating agents, low molecular weight proteins or other stabilizers or excipients. One skilled in the art would know that the choice of a pharmaceutically acceptable carrier, including a

physiologically acceptable compound, depends, for example, on the route of administration of the MC receptor ligand and on the particular physico-chemical characteristics of the specific MC receptor ligand.

The invention further relates to methods of administering a pharmaceutical composition comprising an MC receptor ligand such as HP 467 to a subject in order to restrain pathologically elevated cytokine activity in the subject. For example, HP 467 can be administered to a subject as a treatment for inflammation, pain, cachexia and patho-immunogenic diseases such as rheumatoid arthritis, inflammatory bowel disease and systemic lupus erythematosus, each of which is characterized by pathologically elevated cytokine activity. As used herein, the term "pathologically elevated" means that a cytokine activity is elevated above a range of activities that is expected in a normal population of such subjects. For example, a normal range of IL-1 activity present in a specific tissue can be determined by sampling a statistically significant number of normal, healthy subjects in the population. A subject having a pathology characterized by cytokine-induced pathological effects can be identified by determining that the cytokine activity in the subject is pathologically elevated above the normal range. In particular, a pathologically elevated level of cytokine activity is at least about one standard deviation above the normal, and can be two standard deviations or greater above the normal range.

A pharmaceutical composition comprising an MC receptor ligand such as HP 467 can be administered to a subject having pathologically elevated cytokine activity by various routes including, for example, orally, intravaginally, rectally, or parenterally, such as

intravenously, intramuscularly, subcutaneously, intraorbitally, intracapsularly, intraperitoneally, intracisternally or by passive or facilitated absorption through the skin using, for example, a skin patch or transdermal iontophoresis, respectively. Furthermore, the composition can be administered by injection, intubation or topically, the latter of which can be passive, for example, by direct application of an ointment or powder, or active, for example, using a nasal spray or inhalant. A cytokine restraining agent also can be administered as a topical spray, in which case one component of the composition is an appropriate propellant. The pharmaceutical composition also can be incorporated, if desired, into liposomes, microspheres or other polymer matrices (Gregoriadis, Liposome Technology, Vols. I to III, 2nd ed., CRC Press, Boca Raton, FL (1993), which is incorporated herein by reference). Liposomes, for example, which consist of phospholipids or other lipids, are nontoxic, physiologically acceptable and metabolizable carriers that are relatively simple to make and administer.

The invention also provides methods of assaying for the presence of an MC receptor in a sample by contacting the sample with a radiolabeled peptide, for example, ^{125}I-HP 467. The method further consists of removing unbound radiolabeled peptide from the sample and determining the binding of the radiolabeled peptide. In addition to labeling the peptide with a radioactive moiety, the peptide also can be modified to introduce a chemical moiety that can be readily detected, for example, a fluorescent moiety, as long as the introduced chemical moiety does not interfere with binding of the peptide to one or more MC receptors.

The sample is contacted with the radiolabeled peptide under conditions that allow specific binding of the peptide to the sample. One skilled in the art will know or can readily determine conditions that allow specific binding of the radiolabeled peptide to the sample. Such conditions include, for example, temperature, pH and time of incubation. For example, the conditions that allow specific binding of a peptide to a sample are generally about physiological pH, at a temperature between about 4°C and 37°C, and for a time of about 30 min to 16 hr. Such conditions include, for example, those disclosed in Examples III and IV.

Binding assays of MC receptor transfected cell lines as well as a receptor binding assay in rat brain homogenates have been developed. Initial experiments using a tritiated ligand failed to yield specific binding, so the disclosed assay of the invention was developed using ^{125}I-HP 467. Mouse L cells and human embryonic kidney (HEK) 293 cell lines were transfected with various MC receptors to determine if HP 467 displayed specificity for MC receptor types. Advantages of HEK 293 cells over L cells for MC receptor assays include the high receptor numbers per cell for all receptors including MC1, tight Scatchard plots for all receptors including MC1, and the human cell origin of the HEK 293 cell lines expressing human receptors, which can reflect MC receptor binding in human better than cell lines from other species.

Binding kinetics and competition with standard analogues confirmed that the binding site of HP 467 is an MC receptor (see Examples III and IV). Thus, the present invention provides a new radioligand for MC receptors,

Ac-Nle-Gln-His-(p(^{125}I)-D-Phe)-Arg-(D-Trp)-Gly-NH_2 (^{125}I-HP 467).

The effects of HP 467 on cytokines, via its binding to MC receptors, are similar to those for HP 228 (see Examples V through IX). The effect of HP 228 on cytokines and the uses provided thereby are described, for example, in U.S. Patent No. 5,420,109, WO 95/13086 and WO 96/27386, each of which is incorporated herein by reference. The present invention provides a method of restraining a pathologically elevated cytokine activity in a subject by administering to the subject an effective amount of HP 467. The pathologically elevated cytokine activity can be due, for example, to inflammation, cachexia, or a patho-immunogenic disease. Interestingly, however, HP 467 antagonizes HP 228 induced hypophagia and acute metabolic effects caused by HP 228 (see Examples VIII and IX). Therefore, HP 467 can additionally be used to antagonize HP 228 in the areas of obesity (food intake) and metabolism. In addition, HP 467 can be used as a lead compound for new drug discovery related to antagonism of known MC agonists.

Cytokine expression can result in damage to healthy tissue in a subject and, in extreme cases, can lead to severe disability and death. Cytokines can be expressed at a site of localized infection or can be expressed systemically, for example, in an immune response or in response to bacterial endotoxin-induced sepsis. Cytokine expression can induce pyrexia (fever) and hyperalgesia (extreme sensitivity to pain) in a subject, as well as macrophage and monocyte activation, which produces or further contributes to an inflammatory response in a subject.

Since cytokine expression can be localized or systemic, one skilled in the art would select a particular route and method of administration of HP 467 based on the source and distribution of cytokines in a subject. For example, in a subject suffering from a systemic condition such as bacterial endotoxin-induced sepsis, a pharmaceutical composition comprising HP 467 can be administered intravenously, orally or by another method that distributes the compound systemically. However, in a subject suffering from a pathology caused by localized cytokine expression such as acute respiratory distress syndrome, HP 467 can be suspended or dissolved in the appropriate pharmaceutically acceptable carrier and administered directly into the lungs using a nasal spray or other inhalation device.

In order to restrain the biological activity of a cytokine, HP 467 must be administered in an effective dose, which is about 0.01 to 100 mg/kg body weight. The total effective dose can be administered to a subject as a single dose, either as a bolus or by infusion over a relatively short period of time, or can be administered using a fractionated treatment protocol, in which the multiple doses are administered over a more prolonged period of time. One skilled in the art would know that the concentration of HP 467 required to obtain an effective dose in a subject depends on many factors including the age and general health of the subject as well as the route of administration and the number of treatments to be administered. In view of these factors, the skilled artisan would adjust the particular dose so as to obtain an effective dose for restraining cytokine activity.

Examples of the effectiveness of HP 467 in preventing or minimizing adverse biological effects mediated by cytokines are provided in Examples V through IX and summarized in Table IV. HP 467 can effectively restrain cytokine expression in mice and provide relief from cytokine-mediated swelling and lethality. Thus, HP 467 can be used as a medicament for the treatment of pathologies such as inflammation, pain, cachexia and patho-immunogenic diseases such as arthritis, inflammatory bowel disease and systemic lupus erythematosus, which are characterized by altered cytokine activity.

The following examples are intended to illustrate but not limit the invention.

EXAMPLE I

Synthesis of HP 467

This example provides a standard preparation of HP 467.

HP 467 was synthesized essentially as described in United States Patent No. 5,420,109. Briefly, 100 mg MBHA resin containing a t-Boc Gly derivative was added to a reaction vessel suitable for solid phase peptide synthesis (Houghten, Proc. Natl. Acad. Sci. USA 82:5131 (1985), which is incorporated herein by reference). The following conditions were used for peptide synthesis: coupling was performed in 6-fold excess in N,N-dimethylformamide (DMF) with 0.2 M N-hydroxybenzotriazole(HOBt) and 0.2 M N,N-diisopropylcarbodiimide (DIC) for 90 minutes; activation was performed with 5% diisopropylethylamine (DIEA) in methylene chloride (DCM) for three washes of

2 min; deprotection was performed with 55% trifluoroacetic acid (TFA) for 30 min; washes were performed with DCM and isopropyl alcohol (IPA); the ninhydrin test was run after washing with DMF, DCM and methanol; acetylation was performed with acetylimidazole in 40-fold excess DCM for 4 hr; and cleavage was performed with hydrofluoric acid (HF) and anisole for 90 min.

Peptide synthesis was carried out with the sequential steps of activation, coupling of amino acid, ninhydrin test, deprotection and washing, and the steps were repeated for addition of a new amino acid at each cycle. The amino acids were coupled in the order D-Trp, L-Arg, *p*-iodo-D-Phe, L-His, L-Gln and L-Nle. The peptide was acetylated and the DNP protecting group was removed from His using 2.5% thiophenol in DMF followed by removal of formyl protecting groups in 25% HF in dimethyl-sulphide. The peptide was cleaved from the resin and processed as described previously (United States Patent No. 5,420,109). The resulting peptide was approximately 80 to 90% pure.

EXAMPLE II

Peptide Iodination

This example provides methods for iodinating a peptide.

For diazotization, 1.0 ml 2 N H_2SO_4 was added to 3.0 mg peptide containing *p*-NH_2-Phe. A 6.67 µl aliquot (0.02 µmol) was transferred to a reaction vial, and 79.3 µl of 2 N H_2SO_4 was added. A 6.90 µl aliquot (0.02 µmol) of 3 mM $NaNO_2$ was added, and the reaction was incubated at 0°C for 30-40 min.

For iodination, 100 µl 2 N H_2SO_4 and 400 µl 0.5 M $CuSO_4$ was added to 12.0 mg Zn powder, and the components were allowed to react with periodic mixing for 30-45 min, with venting, until bubbling stopped. The grains were washed twice with H_2O. For unlabeled peptide, 7.12 µl of 0.67 mM NaI (0.0047 µmol) was added to the reaction vial. For radiolabeled peptide, 0.0047 µmol of $Na^{125}I$ was added to the reaction vial. Approximately 1/8 of the copper grains was added to the vial, and the vial was vortexed 1 min. The reaction was carried out vented at room temperature for 3 hr with periodic mixing.

Samples were analyzed on a Vydac 218TP54 C-18 column and were monitored at 214 nm. Buffer A was 0.05% TFA in H_2o, and Buffer B was 0.05% TFA in acetonitrile. Samples were resolved using a 2% per minute gradient from 5 to 55% Buffer B in 25 min.

Using the method described in this example, ^{125}I-HP 467 was routinely labeled to a specific activity of 2000 Ci/mmol. These results demonstrate that HP 467 can be iodinated to generate unlabeled iodo-peptide or high specific activity radiolabeled iodo-peptide.

EXAMPLE III

Melanocortin Receptor Binding Assay Using ^{125}I-HP 467 In Rat Brain Homogenate

This example provides a binding assay using ^{125}I-HP 467 to detect MC receptors in rat brain homogenate.

For assays, fine chemicals were obtained from Sigma (St. Louis MO) and GF/B plates and MICROSCINT were obtained from Packard Instrument Co. (Meriden CT).

Frozen rat brains were thawed and the thalamus and hypothalamus were dissected out and weighed. The tissue was homogenized in 40 ml buffer A (50 mM Tris-HCl, 2 mM EDTA, 10 mM $CaCl_2$, 100 µM PMSF, pH 7.4) in a Dounce homogenizer. The homogenate was centrifuged at 39,000 xg for 10 min, resuspended in 20 ml fresh buffer and recentrifuged. The pellet was resuspended in 80 ml buffer B (50 mM Tris-HCl, 2 mM EDTA, 10 mM $CaCl_2$, 5 mM $MgCl_2$, 100 µM PMSF, pH 7.4). ^{125}I-HP 467 was custom labeled by Amersham to a specific activity of 2000 Ci/mmol (Amersham; Arlington Heights IL). Fifty pM ^{125}I-HP 467 and 0.2 mg protein/ml membrane suspension containing 2 mg/ml BSA was added to each assay tube.

Typical assay volumes were 50 µl ^{125}I-HP 467, 50 µl HP 228 and 250 µl membrane. Competition assays were performed using α-MSH, γ1-MSH, γ2-MSH, γ3-MSH and ACTH (4-10) also containing 1 mM phenanthroline, 200 µg/ml bacitracin, and 5 µg/ml leupeptin. Tubes were incubated for 2 hours at 37°C. The assay was terminated by filtration through GF/B filters previously soaked in 5 mg/ml BSA Tris-HCl buffer. The samples were washed with Tris-HCl, dried and counted in Packard Minaxl gamma counter (Packard Instrument Co.).

Binding was found to be tissue specific with most binding observed in rat hypothalamus. As shown in Figures 1 to 4, binding of ^{125}I-HP 467 was saturable. Saturation binding curves of ^{125}I-HP 467 bound to rat brain membranes indicated a Kd of 0.4 nM and a Bmax of 21 fmoles/mg protein.

These results demonstrate that ^{125}I-HP 467 can be used in a binding assay to detect MC receptors in rat brain homogenates.

EXAMPLE IV

Melanocortin Receptor Binding Assay Using ^{125}I-HP 467 in Transfected Cells

This example demonstrates the use of ^{125}I-HP 467 for assaying MC receptor binding in human and mouse cell lines transfected to express MC receptors.

All cell culture media and reagents were obtained from GibcoBRL (Gaithersburg MD), except for COSMIC CALF SERUM (HyClone; Logan UT). HEK 293 and mouse L cell lines were transfected with the human MC receptors hMC1, hMC3, and hMC4 (Gantz et al., Biochem. Biophys. Res. Comm. 200:1214-1220 (1994); Gantz et al., J. Biol. Chem. 268:8246-8250 (1993); Gantz et al. J. Biol. Chem. 268:15174-15179 (1993); Haskell-Leuvano et al., Biochem. Biophys. Res. Comm. 204:1137-1142 (1994); each of which is incorporated herein by reference). Vectors for construction of an hMC5 expressing cell line were obtained, and a line of HEK 293 cells expressing hMC5 was constructed (Gantz, *supra*, 1994). hMC5 has been described previously (Franberg et al., Biochem. Biophys. Res. Commun. 236:489-492 (1997); Chowdhary et al., Cytogenet. Cell Genet. 68:1-2 (1995); Chowdhary et al., Cytogenet. Cell Genet. 68:79-81 (1995), each of which is incorporated herein by reference). L cell lines were maintained in MEM containing 25 mM HEPES, sodium pyruvate, 10% COSMIC CALF SERUM, 100 units/ml penicillin, 100 µg/ml streptomycin, and 0.2 mg/ml G418 to maintain selection. For HEK 293 cells, DMEM was used instead of MEM, and 2 mM glutamine, non-essential amino acids, and vitamins were included in addition to the above mentioned additives.

Before assaying, cells were washed once with phosphate buffered saline ("PBS"; without Ca^{2+} and Mg^{2+}), and stripped from the flasks using 0.25% trypsin and 0.5 mM EDTA. Cells were suspended in PBS, 10% COSMIC CALF SERUM and 1 mM $CaCl_2$. Cell suspensions were prepared at a density of $2x10^4$ cells/ml for HEK 293 cells expressing hMC3, hMC4 or hMC5, and $1x10^5$ cells/ml for HEK 293 cells expressing hMC1. For L cells, cells expressing MC3 or MC4 were suspended at $2x10^5$ cells/ml, and cells expressing MC1 suspended at $8x10^5$ cells/ml. Suspensions were placed in a water bath and allowed to warm to 37°C for 1 hour.

Binding assays were performed in a total volume of 250 µl for HEK 293 cells, and a volume of 600 µl for L cells. Peptides and other compounds were dissolved in distilled water. ^{125}I-HP 467 (2000 Ci/mmol) was prepared in 50 mM Tris, pH 7.4, 2 mg/ml BSA, 10 mM $CaCl_2$, 5 mM $MgCl_2$, 2 mM EDTA and added to each tube, with 50,000 dpm for HEK 293 assays or 100,000 dpm for L cell assays. To each tube was added $4x10^3$ HEK 293 cells expressing hMC3, hMC4 or hMC5, or $2x10^4$ cells expressing hMC1. For L cells expressing hMC3 or hMC4, $1x10^5$ cells were used, and for L cells expressing hMC1, $4x10^5$ cells were used. Assays were incubated for 2.5 hr at 37°C.

GF/B filter plates were prepared by soaking for at least one hour in 5 mg/ml BSA and 10 mM $CaCl_2$. Assays were filtered using a Brandel 96-well cell harvester (Brandel Inc.; Gaithersburg, MD). The filters were washed four times with cold 50 mM Tris, pH 7.4, the filter plates were dehydrated for 2 hr and 35 µl of MICROSCINT was added to each well. Filter plates were counted using a Packard Topcount (Packard Instrument Co.) and data analyzed using GraphPad PRISM v2.0 (GraphPad

Software Inc.; San Deigo CA) and Microsoft EXCEL v5.0a (Microsoft Corp.; Redmond WA).

Binding assays were performed in duplicate in a 96 well format utilizing a 1.2 ml cluster tube system (Corning Costar; Cambridge MA). HP 467 was prepared in 50 mM Tris, pH 7.4, and ^{125}I-HP 467 was diluted to give 100,000 dpm per 50 µl. Serial dilutions (10-fold or 5-fold) of HP 228, α-MSH, NDP and unlabeled HP 467 were prepared from 1.2 mM stock solutions. All assay tubes contained 50 µl of ^{125}I-HP 467 and 50 µl of either Tris buffer (for determination of total binding) or diluted peptide (serial dilutions of HP 228, α-MSH, NDP or unlabeled HP 467).

Saturation binding experiments were performed in duplicate in the same volume with the same number of cells as described above for the binding assay. Saturation radioligand binding experiments measure specific radioligand binding at equilibrium at various concentrations of the radioligand. Scatchard analysis of the data was used to determine receptor number and ligand affinity. ^{125}I-HP 467 was added to the assay, with 2×10^8 dpm being the highest concentration and 2-fold serial dilutions made thereafter to obtain 8 to 12 data points. Nonspecific binding was determined in the presence of unlabeled HP 467 at 1000 times the concentration of the ^{125}I-HP 467. The actual amount of tracer added was determined by gamma counting the test tubes on a Packard Minaxl gamma counter. The concentration of ^{125}I-HP 467 in the assay was calculated from the half-life corrected dpm using EXCEL v5.0a.

The results of displacement binding assays using L cells for MC receptors hMC1, hMC3, and hMC4 are

summarized in Table III. Saturation curves for the L cells are shown as Figures 5, 6 and 7 for MC1, MC3 and MC4, respectively. The K_i apparent and displacement binding assays determined from receptor binding assays for hMC5 in HEK 293 cells also are included in Table III. Saturation binding isotherms and Scatchard plots for MC1, MC3 and MC4 in HEK 293 cells are included as Figures 8, 9 and 10, respectively. BMAX is indicated as receptors/cell and KD is nM.

As shown in Figures 5 through 10, ^{125}I-HP 467 can bind to the MC receptors MC1, MC3 and MC4. As shown in Table III, ^{125}I-HP 467 also binds to the MC5 receptor. The binding of ^{125}I-HP 467 can be displaced by various MC receptor ligands with differing IC_{50} values depending on the specific MC receptor expressed in the cell line.

TABLE III - DISPLACEMENT OF ^{125}I-HP 467					
PEPTIDE		RECEPTOR			
		MC-1	MC-3	MC-4	MC-5
HP 228	Binding IC_{50}	1.49	29.70	7.51	194.50
	S. Dev.	0.78	5.23	2.95	78.49
	n	3	4	4	2
HP 467	Binding IC_{50}	0.23	0.48	0.31	0.46
	S. Dev.	0.17	0.25	0.20	0.25
	n	11	15	14	6
α-MSH	Binding IC_{50}	9.31	21.52	98.12	
	S. Dev.	6.41	7.92	30.24	
	n	4	6	5	0
NDP	Binding IC_{50}	1.90	2.01	4.36	5.85
	S. Dev.	1.44	1.53	2.79	1.92
	n	5	5	6	2

"S. Dev." is the Standard Deviation; "n" represents the number of samples.

MC1, MC3 and MC4 receptors were expressed in mouse L cells and MC5 in HEK 293 cells. Concentrations ranged from 10^{-5} to 10^{-14} M.

EXAMPLE V

Reduction of Lipopolysaccharide-Induced Tumor Necrosis Factor Levels in Mice

This example describes the effectiveness of HP 467 for decreasing tumor necrosis factor (TNF) levels in lipopolysaccharide (LPS; endotoxin) treated mice.

BALB/c female mice weighing approximately 20 g were placed into a control group and three HP 467

treatment groups. Five mg/kg of LPS in 0.9% saline was administered by intraperitoneal (IP) injection to all mice. Mice in the treatment group received either 30, 150 or 300 µg of HP 467 per mouse in a volume of 100 µl. Control mice received 100 µl of saline alone. One minute after initial injections all mice received the LPS injection.

Blood samples were collected from the orbital sinus of treated and control mice 90 minutes after LPS administration. The plasma was separated by centrifugation at 3000 x g for 5 min and stored at -20°C. Samples were thawed and diluted with four volumes of 1x PBS (pH 7.4) containing 1% bovine serum albumin. A 100 µl sample of plasma was assayed by ELISA for TNF-α (Genzyme; Cambridge MA).

The mean (± SEM) TNF-α level in five mice from each group was determined and the percent reduction in TNF-α levels was calculated. As shown in Table IV, treatment of mice with HP 467 decreased the levels of TNF-α in a dose dependent manner when compared to saline controls with a 3% decrease observed with 30 µg/mouse, a 78% decrease with 150 µg/mouse and an 81% decrease with 300 µg/mouse.

These results indicate that HP 467 can restrain LPS-induced cytokine activity.

TABLE IV - BIOLOGICAL DATA FOR HP 467

Biological Test	Dose	Efficacy
Reduction in TNF levels	30 µg/mouse	3%
	150 µg/mouse	78%
	300 µg/mouse	81%
Inhibition of LPS-Induced lethality	300 µg/mouse BID	50%
	500 µg/mouse TID	40%
Reduction in arachidonic acid-induced ear swelling	100µg/mouse	82%

	Antagonism of HP 228 Drug/Dose	Change Day 1	Change Day 2
Hypophagia (food intake)	Saline	+5.3%	+12.1%
	HP 228 (1.5 mg/Kg)	-6.8%	-5.3%
	HP 228/HP 467	+2.1%	+4.8%
Oxygen Consumption	HP 228/HP 467 (5 mg/kg / 10 mg/kg)	50% of HP 228	

EXAMPLE VI

Lipopolysaccharide-Induced Lethality

This example describes the effectiveness HP 467 in reducing lethality from sepsis induced by administration of LPS.

These experiments were performed based on information reported by Rivier et al., Endocrinology 125:2800-2805 (1989), which is incorporated herein by reference. Adult female BALB/c mice were provided food and water *ad libitum*. Mice were treated either IP every 8 hours (three times a day; TID) for 40 hours with 500 µg HP 467 in 200 µl saline or at 8 AM and 4 PM (twice a day; BID) for 40 hours with 300 µg of HP 467 in 200 µl saline. Control animals received injections of saline at the respective times of HP 467 treatment. Immediately following the first injection, 0.6 mg LPS endotoxin in 200 µl saline was administered to each mouse. All groups contained 10 mice.

As shown in Table IV, administration of HP 467 in both dosing regimens significantly increased survival as compared to the saline control mice. There was a 50% survival rate with the BID treatment regimen using HP 467 (300 µg/mouse) and a 40% survival rate with the TID treatment regiment using HP 467 (500 µg/mouse). All mice in the saline control group died within the 40 hours of the study (100% mortality).

These results show that HP 467 significantly inhibits LPS-induced lethality.

EXAMPLE VII

Reduction of Arachidonic Acid-Induced Ear Swelling in Mice

This example demonstrates that HP 467 can reduce arachidonic acid-induced ear swelling in mice.

Experiments were performed using female BALB/c mice weighing 18 to 23 grams. Saline or 100 µg HP 467

was administered IP, 30 minutes prior to topical application of arachidonic acid (Calbiochem-Novabiochem; San Diego CA). A 10 µl pipette was used to apply 10 µl of a 100 mg/ml arachidonic acid solution in acetone to the inner and outer surfaces of the right ear of each mouse. Ten ml of acetone (alone) was applied to the inner and outer surface of the left ear of each mouse.

Ear thickness was measured with a hand held spring loaded caliper 60 minutes after arachidonic acid application. Increase in ear thickness was calculated by subtracting the thickness of the control ear from the thickness of the arachidonic acid-treated ear. The value for each group is the average of the swelling observed in the mice of each group. The percent reduction in swelling is based on the swelling observed in the saline control group. As shown in Table IV, HP 467 reduced the level of arachidonic acid-induced ear swelling by 81%.

These results show that HP 467 significantly reduces arachidonic acid-induced ear swelling.

EXAMPLE VIII

HP 467 Antagonism of HP 228 Induced Hypophagia

HP 228 reduces body weight (see WO 96/27386). This example shows that HP 467 antagonizes the effect of HP 228-induced hypophagia, thereby reversing the undesirable decrease in food consumption that occurs following HP 228 administration.

Male Sprague-Dawley rats weighing 250 to 300 grams were divided into three treatment groups of 8 rats each. The control group received IP saline injections BID (1 ml/kg) at 8 AM and 4 PM. Two groups of rats

received HP 228 injections IP (1.5 mg/kg) at the same dosing schedule as the control group. After each injection, each rat immediately received a second injection. The second injection for the saline group and one HP 228 group was another injection of saline (1 ml/kg), and the second injection for the remaining HP 228 group was HP 467 at a dose of 3.0 mg/kg. All injections started at 4 PM on day 0 and ended on the afternoon of day 2. Food consumption and body weight measurements were taken daily, each morning.

As shown in Table IV, food intake in the saline control group increased daily as expected. HP 228 treatment caused a decrease in food consumption during the study period, and HP 467 reduced the decrease in food consumption caused by administration of HP 228. These results demonstrate that HP 467 antagonizes the effect of HP 228-induced hypophagia.

EXAMPLE IX

HP 467 Antagonism of Acute Metabolic Effects by HP 228

Weight loss observed with HP 228 correlates to an increased metabolic rate as determined by increased resting oxygen consumption (see WO 96/27386). This example shows that HP 467 antagonizes the acute metabolic effects caused by HP 228 administration.

Male Sprague-Dawley rats (250 to 350 grams) were divided into three treatment groups for the measurement of acute metabolic effects. All injections were administered IP. PBS (1 ml/kg) was administered to two of the groups. The third group received 10 mg/kg HP 467. Fifteen minutes after the initial injections, one PBS group received another injection of PBS (1 ml/kg)

and the second group received 5 mg/kg HP 228. The group receiving the initial injection of HP 467 received 5 mg/kg HP 228. Immediately after the second injection the rats were placed into the Oxymax System (Columbus Instruments; Columbus OH) for monitoring. Data was collected 10 minutes after the animal was acclimated to the cage and continued for a total of 50 minutes. The parameters measured were resting oxygen consumption (VO_2), resting respiratory quotient (RQ), and total locomotor activity. As shown in Table IV, HP 467 attenuated the effects of HP 228 on VO_2.

These results demonstrate that HP 467 antagonizes the effect of HP 228 on food intake and oxygen consumption.

EXAMPLE X

Tetrapeptide Ligands for Melanocortin Receptors

This example demonstrates the use of a positional scanning combinatorial library to identify tetrapeptide ligands for MC receptors.

A positional scanning combinatorial library was constructed to contain 91^4 (68,574,960) tetrapeptides of the structure A1-B2-C3-D4. In positional scanning libraries, a defined amino acid is determined for a given position and is "walked" through the length of the peptide, resulting in the defined amino acid appearing in positions A1, B2, C3 and D4. Mixtures active at each of the four positions can be identified in a single screen. The following peptides were synthesized:
Ac-OXXX-NH_2; 91 mixtures each containing 753,571 peptides;
Ac-XOXX-NH_2; 91 mixtures each containing 753,571 peptides;
Ac-XXOX-NH_2; 91 mixtures each containing 753,571 peptides;

Ac-XXXO-NH_2; 91 mixtures each containing 753,571 peptides; where "O" is a defined single amino acid and "X" represents a mixture of 91 L-, D- and amino acids derivatives.

Figure 11 shows the percent bound for all library mixtures prepared from the positional scanning combinatorial library. Table V shows the IC_{50} for the most active mixtures from the screening data. IC_{50} values were determined in brain tissue as described in Example III.

Based on the results shown in Table V, peptides were synthesized with the following amino acids in specific positions in the tetrapeptide: αFmLys and His at position A1; Arg, D-Thi and *p*Cl-f at position B2; Arg, L-Cha and Ile at position C3; and D-Nal and D-Arg at position D4. Synthesis of peptides containing these amino acids resulted in 2 x 3 x 3 x 2 = 36 individual tetrapeptides.

Table Va - IC_{50} values for most active mixtures from the screening data

Library No.	OXXX	IC_{50} µM	Library No.	XOXX	IC_{50} µM
73	αFmLys	88	106	R	14
83	L-hmP	118	168	D-Thi	224
7	H	122	160	*p*Cl-f	235
55	L-Nal	155	116	f	238
15	R	158	125	r	281
34	r	203	141	α-Orn	283
49	ε-Lys	214	161	*p*F-F	335
9	K	220	117	h	363
75	D-pyrala	256	119	k	377
28	k	271	140	ε-Lys	538
26	h	304	142	δ-Orn	563
21	a	467	158	Thiopro	1457
67	Thiopro	549	177	t4-benz	3634
80	L-isoN	765	174	L-hmP	6845
89	3-2Met	2753	182	D-Cit	NA

Table Vb - IC_{50} values for most active mixtures from the screening data

Library No.	XXOX	IC_{50} µM	Library No.	XXXO	IC_{50} µM
197	R	41	328	D-Nal	107
240	L-Cha	59	307	r	135
209	I	105	299	h	209
216	r	142	322	ε-Lys	245
250	*p*Cl-F	164	282	K	546
210	k	190	301	k	559
232	α-Orn	266	297	e	NA
251	*p*Cl-f	648			
217	s	928			
265	L-hmP	1021			
256	L-pyrala	1216			
208	h	1847			
272	Npecot	2213			
229	εAca	3363			
273	D-Cit	NA			
249	Thiopro	NA			

The 36 peptides were screened for MC receptor binding activity in rat brain and in mouse B16 cells. The IC_{50} values for some of these peptides are shown in Table VI. The peptide Ac-His-(*p*Cl-f)-Arg-(D-Nal)-NH_2 was active below 1 µM with an IC_{50} value of 18 nM.

Table VI. Activity of Tetrapeptides on Rat Brain and Mouse B16 Cells

Peptide	Rat Brain IC_{50} (nM)	Mouse B16 IC_{50} (nM)
Ac-H-(*p*Cl-f)-R-(Nal)-NH_2	18	68
Ac-H-(*p*Cl-f)-(L-Cha)-r-NH_2	>5000	156
Ac-(αFmLys)-(*p*Cl-f)-R-(Nal)-NH_2	427	41
Ac-(αFmLys)-R-(L-Cha)-(Nal)-NH_2	>4000	1143
Ac-(αFmLys)-R-(L-Cha)-r-NH_2	427	185
Ac-(αFmLys)-(D-Thi)-R-(Nal)-NH_2	469	>4000
Ac-(αFmLys)-R-R-(Nal)-NH_2	1995	1416
Ac-H-(*p*Cl-f)-R-(D-Nal)-NH_2	51	19

These results show that tetrapeptides that bind to MC receptors were identified from a positional scanning combinatorial library.

Although the invention has been described with reference to the examples provided above, it should be understood that various modifications can be made without departing from the spirit of the invention. Accordingly, the invention is limited only by the following claims.

We claim:

1. A peptide, comprising the sequence:

Ac-Nle-Gln-His-(p(I)-D-Phe)-Arg-(D-Trp)-Gly-NH_2.

2. A composition of matter, comprising a peptide and a pharmaceutically acceptable carrier, said peptide comprising the sequence:

Ac-Nle-Gln-His-(p(I)-D-Phe)-Arg-(D-Trp)-Gly-NH_2.

3. A radiolabeled peptide, comprising the sequence:

Ac-Nle-Gln-His-(p(^{125}I)-D-Phe)-Arg-(D-Trp)-Gly-NH_2.

4. A method of assaying for the presence of a melanocortin receptor in a sample, comprising:

(a) contacting the sample with the radiolabeled peptide of claim 3;

(b) removing unbound radiolabeled peptide from said sample; and

(c) determining the binding of said radiolabeled peptide.

5. The method of claim 4, wherein said sample is a cell.

6. The method of claim 4, wherein said sample is a tissue homogenate.

7. The method of claim 6, wherein said tissue is brain.

8. A method of restraining a pathologically elevated cytokine activity in a subject, comprising administering to the subject an effective amount of the peptide of claim 1.

9. The method of claim 8, wherein said pathologically elevated cytokine activity is due to inflammation.

10. The method of claim 8, wherein said pathologically elevated cytokine activity is due to cachexia.

11. The method of claim 8, wherein said pathologically elevated cytokine activity is due to a patho-immunogenic disease.

12. A peptide, comprising the sequence:

A1-B2-C3-D4, wherein

A1 is αFmLys, L-hmP, His, L-Nal, Arg, D-Arg, ϵ-Lys, Lys, D-pyrala, D-Lys, D-His, D-Ala, Thiopro, L-isoN or 3-2Met;

B2 is Arg, D-Thi, *p*Cl-f, D-Phe, Arg, α-Orn, *p*F-F, D-His, D-Lys, ϵ-Lys, δ-Orn, Thiopro, t4-benz, L-hmP or D-Cit;

C3 is Arg, L-Cha, D-Ile, D-Arg, *p*Cl-F, D-Lys, α-Orn, *p*Cl-f, D-Ser, L-hmP, L-pyrala, D-His, Npecot, ϵAca, D-Cit or Thiopro; and

D4 is D-Nal, D-Arg, D-His, ε-Lys, Lys, D-Lys or D-Glu.

13. The peptide of claim 12, wherein the amino terminus is modified by acetylation.

14. The peptide of claim 12, wherein the carboxy terminus is modified by amidation.

15. The peptide of claim 12, wherein

A1 is αFmLys or His;
B2 is Arg, D-Thi, or *p*Cl-f;
C3 is Arg, L-Cha, or D-Ile; and
D4 is D-Nal or D-Arg.

16. The peptide of claim 15, wherein the amino terminus is modified by acetylation.

17. The peptide of claim 15, wherein the carboxy terminus is modified by amidation.

18. The peptide of claim 15, having the structure selected from the group consisting of:

His-(*p*Cl-f)-(L-Cha)-(D-Arg);
(αFmLys)-(*p*Cl-f)-Arg-(Nal);
(αFmLys)-Arg-(L-Cha)-(Nal);
(αFmLys)-Arg-(L-Cha)-(D-Arg);
(αFmLys)-(D-Thi)-Arg-(Nal);
(αFmLys)-Arg-Arg-(Nal); and
His-(*p*Cl-f)-Arg-(D-Nal).

19. The peptide of claim 18, wherein the amino terminus is modified by acetylation.

20. The peptide of claim 18, wherein the carboxy terminus is modified by amidation.

21. The peptide of claim 15, having the structure His-(*p*Cl-f)-Arg-(D-Nal).

22. The peptide of claim 21, wherein the amino terminus is modified by acetylation.

23. The peptide of claim 21, wherein the carboxy terminus is modified by amidation.

24. A composition of matter, comprising said peptide of claim 12 and a pharmaceutically acceptable carrier.

25. A method of altering the activity of a melanocortin receptor in a subject, comprising administering to the subject an effective amount of the peptide of claim 12.

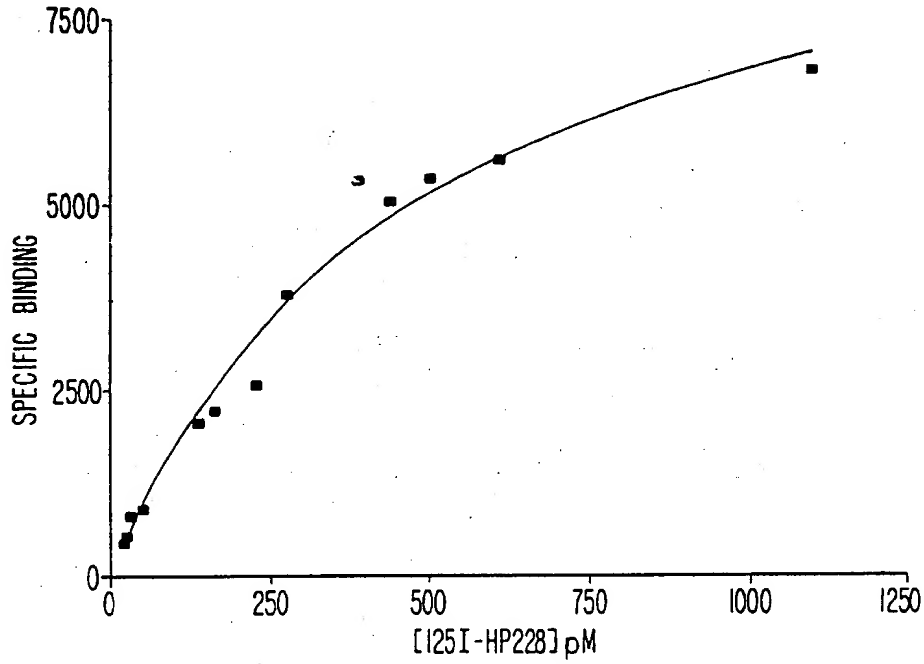

7500
5000
2500
0
SPECIFIC BINDING
0
250
500
750
1000
1250
[125I-HP228] pM

FIG. 1A

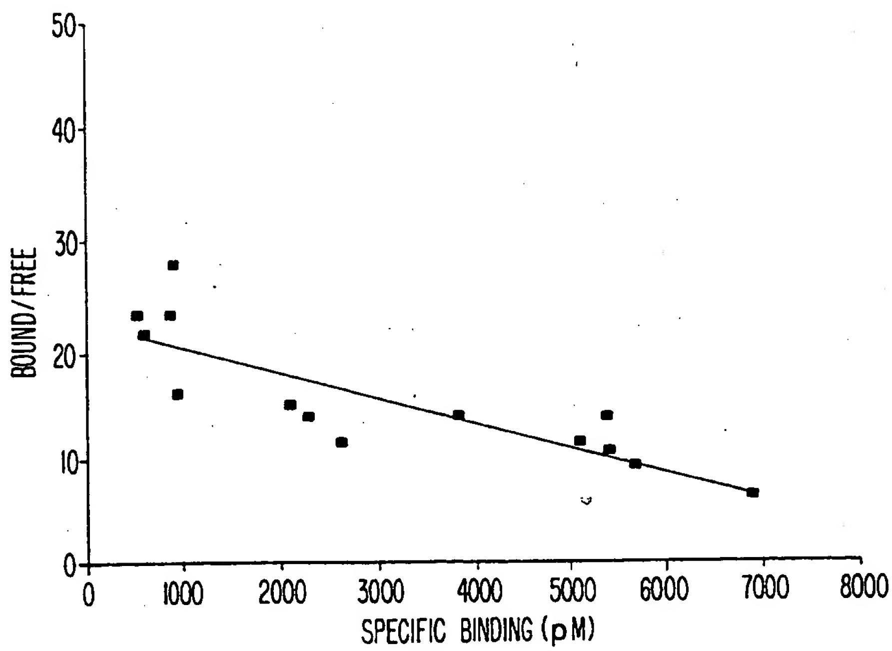

50
40
30
20
10
0
BOUND/FREE
0
1000
2000
3000
4000
5000
6000
7000
8000
SPECIFIC BINDING (pM)

FIG. 1B

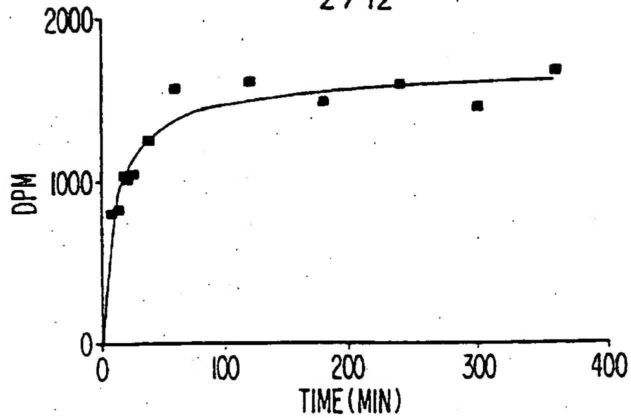


FIG. 2

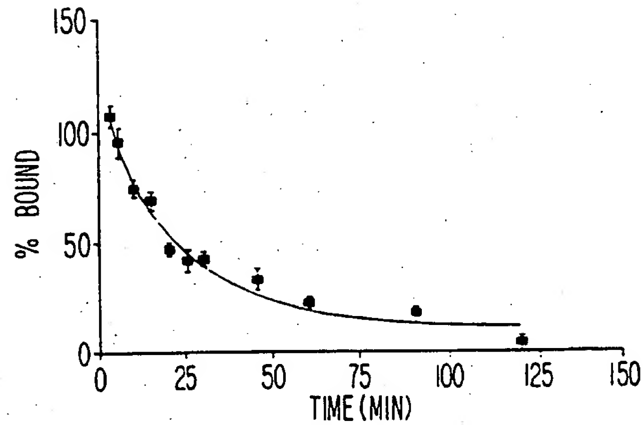


FIG. 3

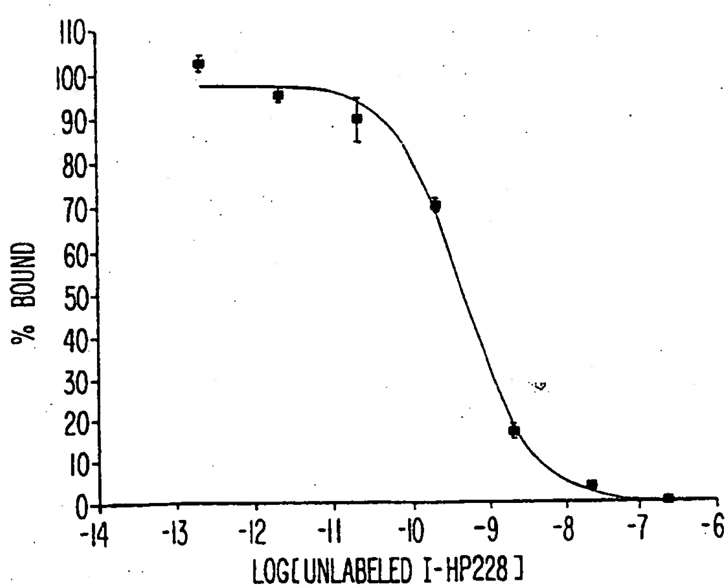
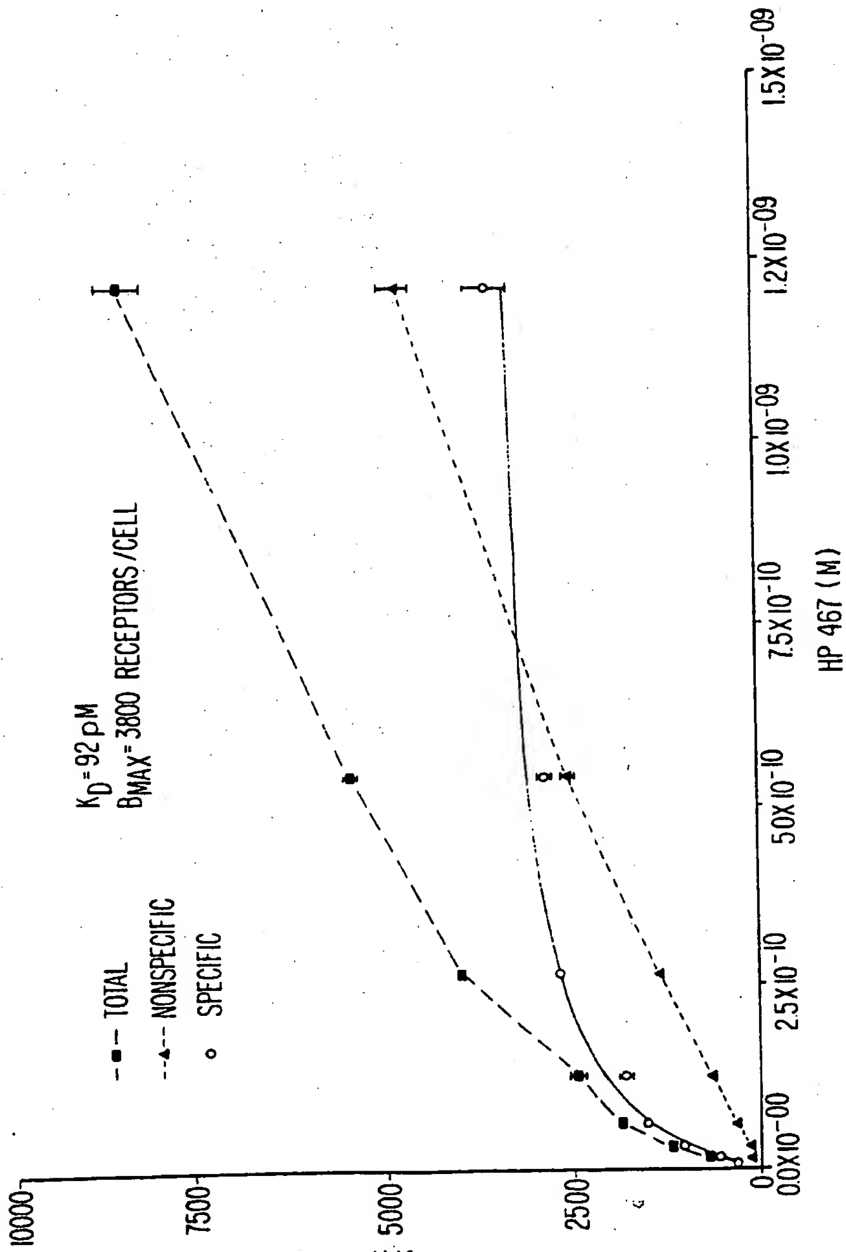
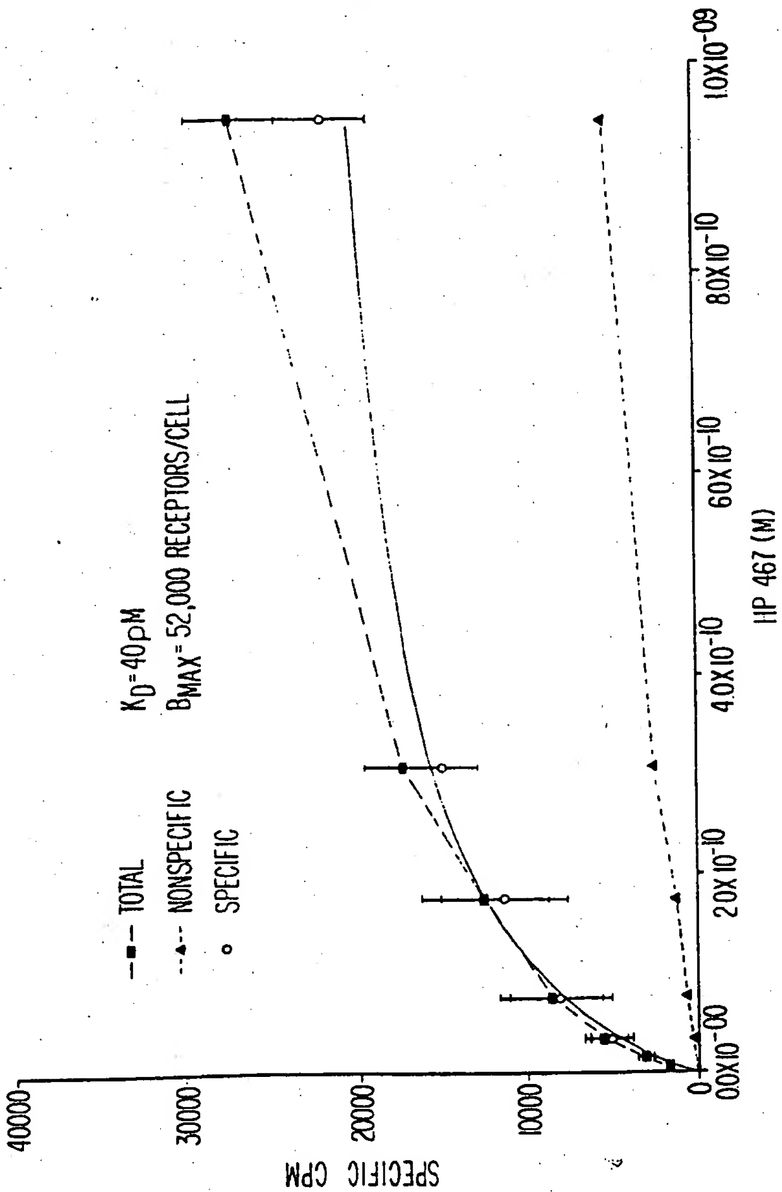


FIG. 4



TOTAL
NONSPECIFIC
SPECIFIC
K_D=92 pM
B_MAX=3800 RECEPTORS/CELL
CPM
10000
7500
5000
2500
0
0.0X10-00
2.5X10-10
5.0X10-10
7.5X10-10
1.0X10-09
1.2X10-09
1.5X10-09
HP 467 (M)

FIG.5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SPECIFIC
K_D=40pM
B_MAX=52,000 RECEPTORS/CELL
SPECIFIC CPM
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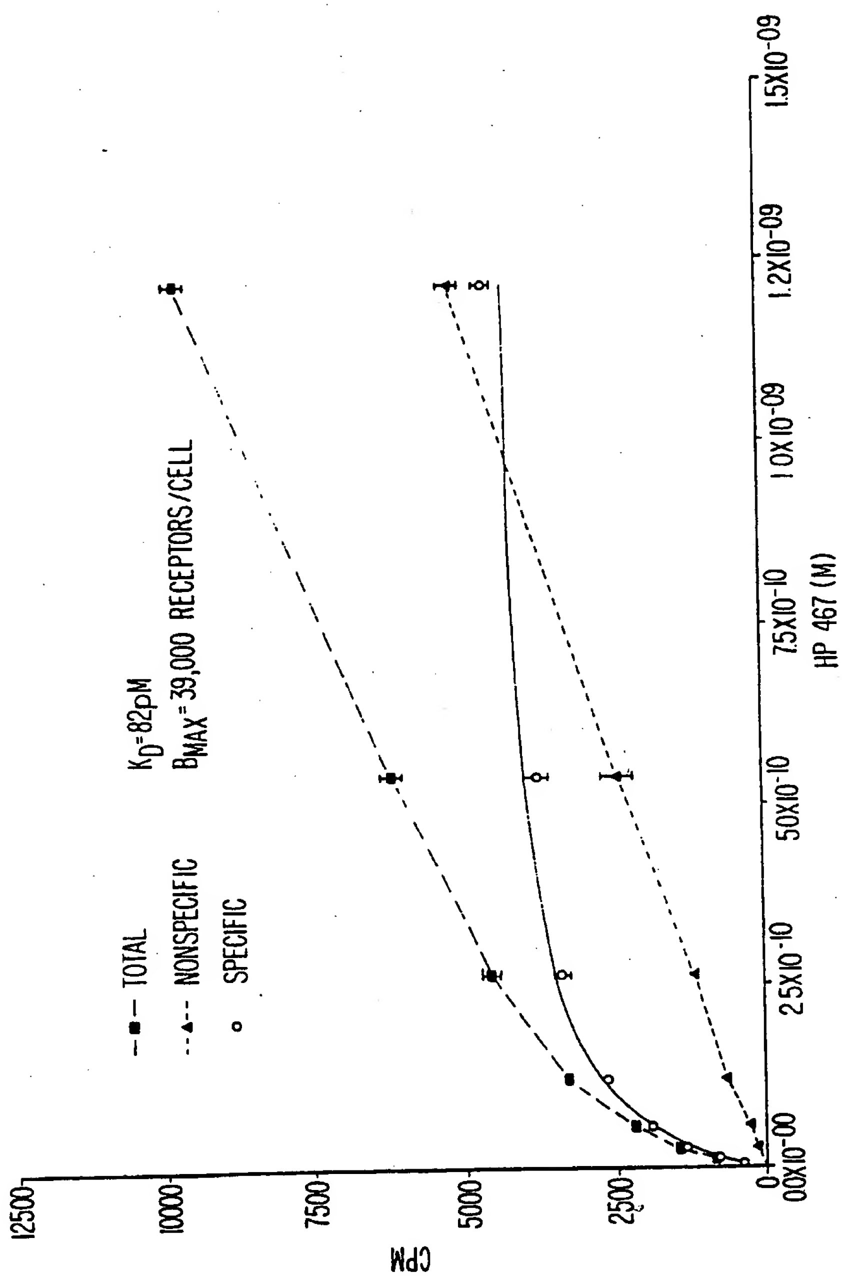
30000
20000
10000
0
0.0X10-00
2.0X10-10
4.0X10-10
6.0X10-10
8.0X10-10
1.0X10-09
HP 467 (M)

FIG.6


TOTAL
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SPECIFIC
K_D=82pM
B_MAX=39,000 RECEPTORS/CELL
CPM
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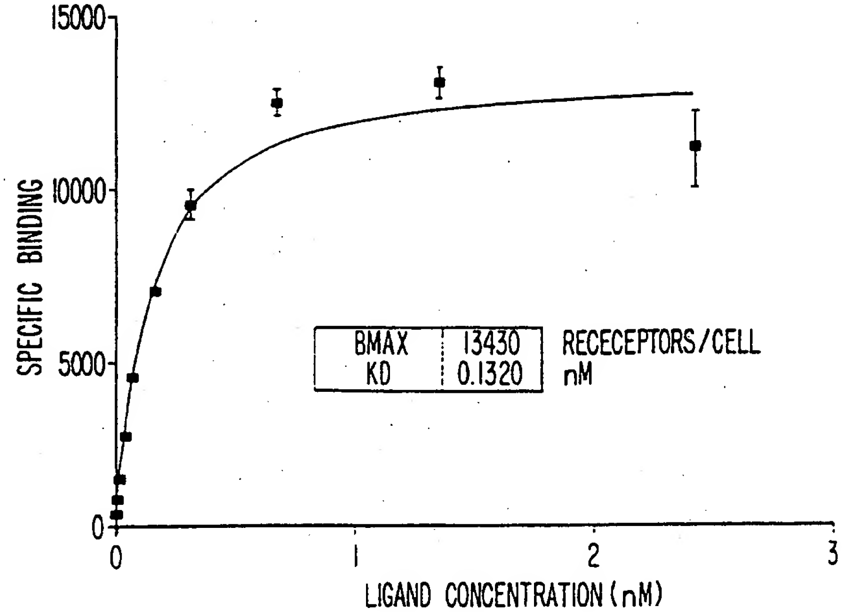
7500
5000
2500
0
0.0X10-00
2.5X10-10
5.0X10-10
7.5X10-10
1.0X10-09
1.2X10-09
1.5X10-09
HP 467 (M)

FIG. 7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10000
5000
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SPECIFIC BINDING
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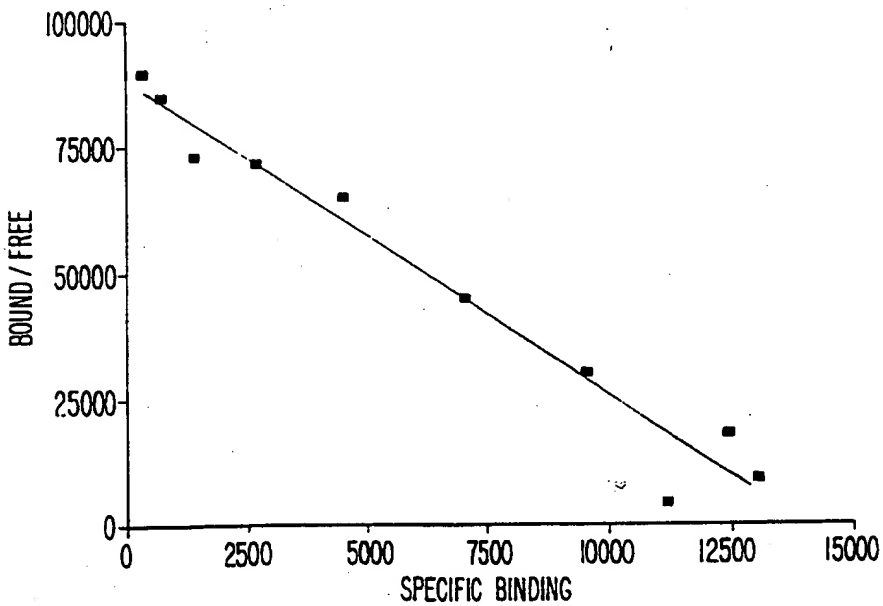
2
3
LIGAND CONCENTRATION (nM)
BMAX 13430 RECECEPTORS/CELL
KD 0.1320 nM

FIG. 8A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75000
50000
25000
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BOUND / FREE
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2500
5000
7500
10000
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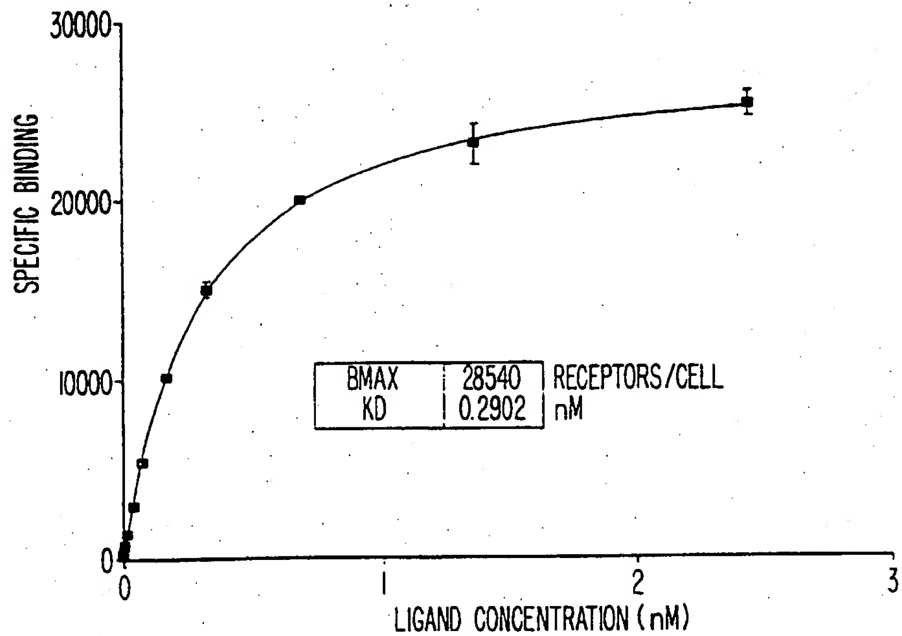
15000
SPECIFIC BINDING

FIG. 8B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SPECIFIC BINDING
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2
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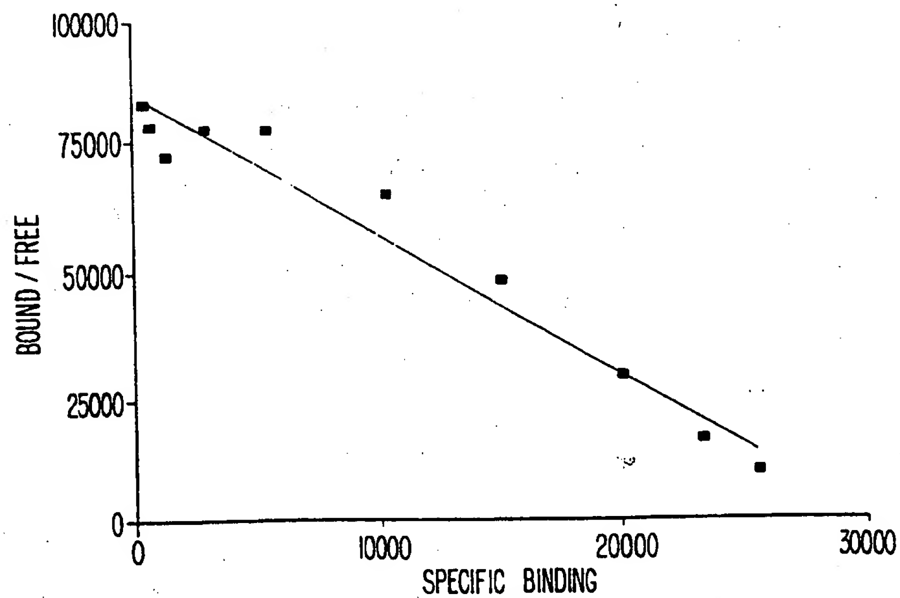
BMAX 28540 RECEPTORS/CELL
KD 0.2902 nM

FIG. 9A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BOUND / FREE
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10000
20000
30000
SPECIFIC BINDING

FIG. 9B

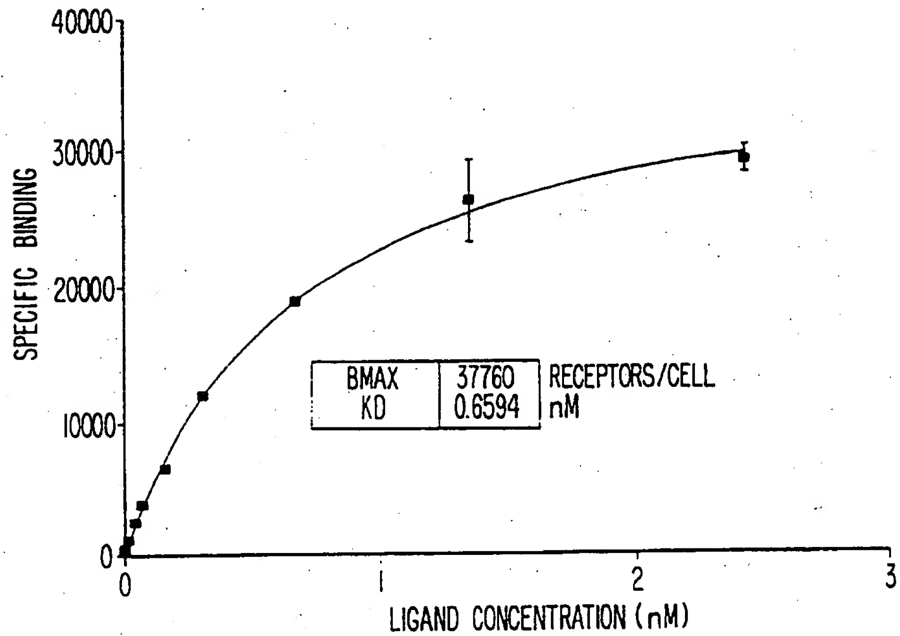


FIG. 10A

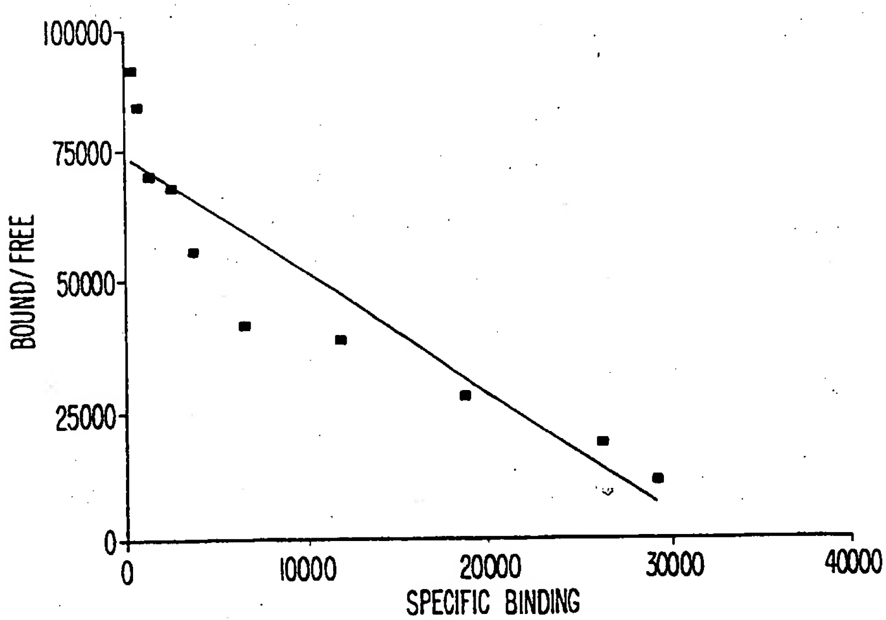


FIG. 10B

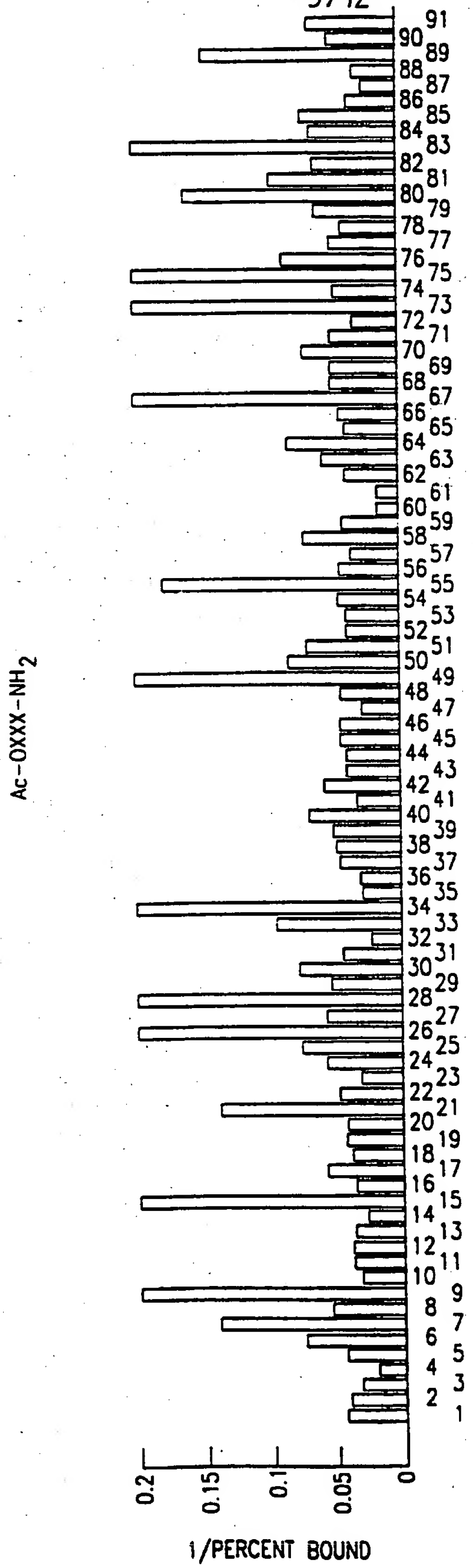


FIG. 11A

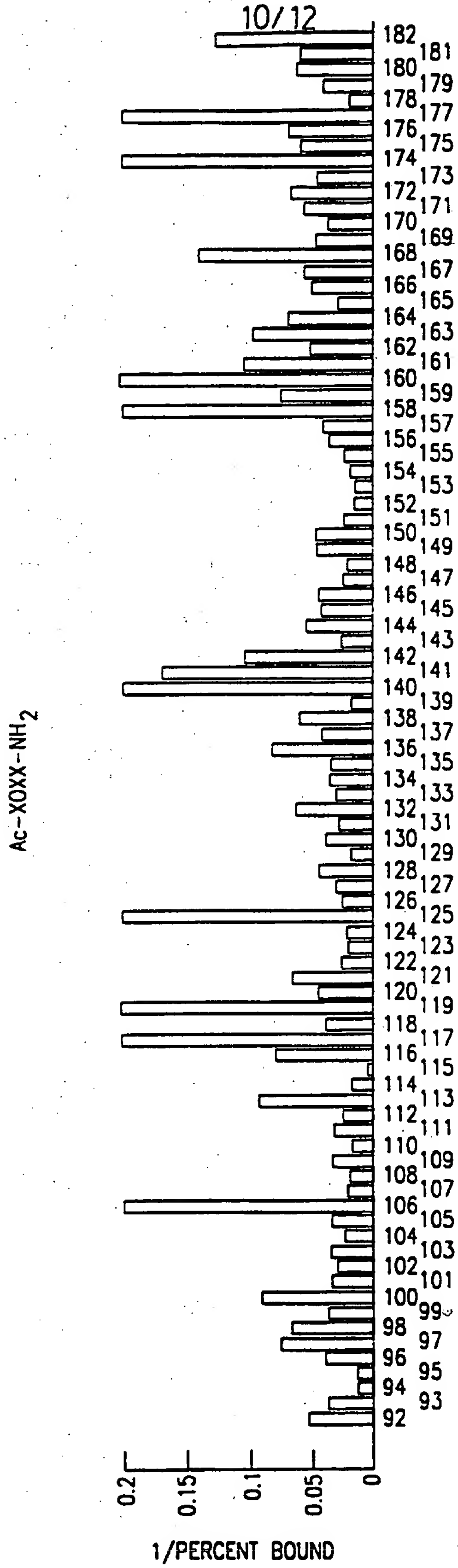


FIG. 11B

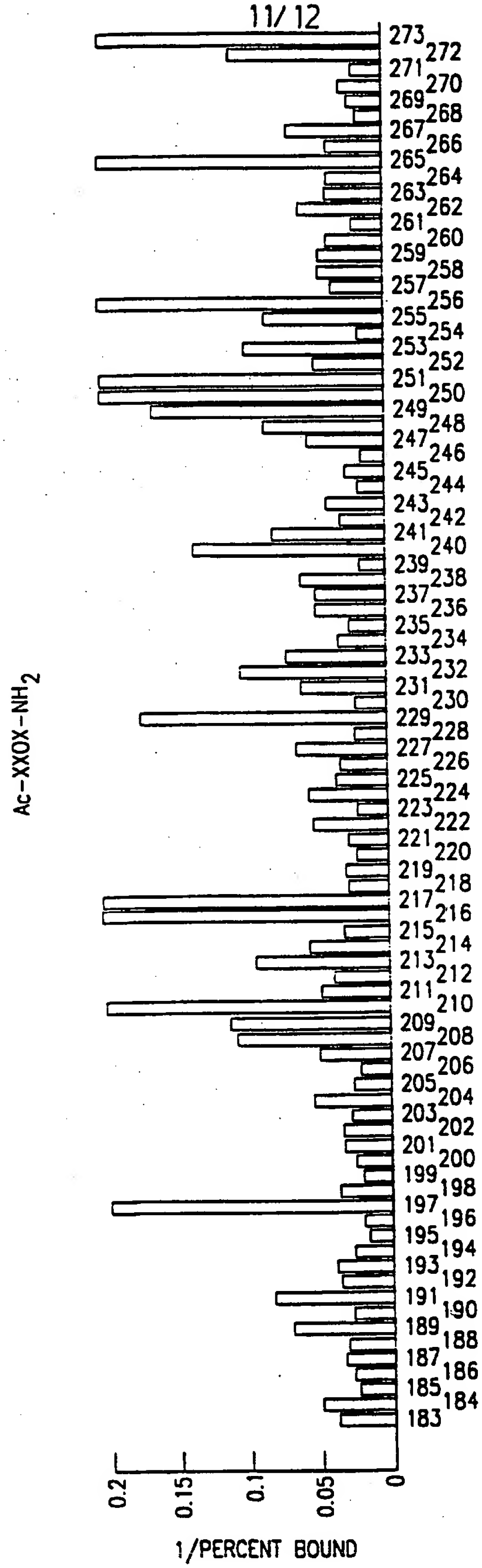
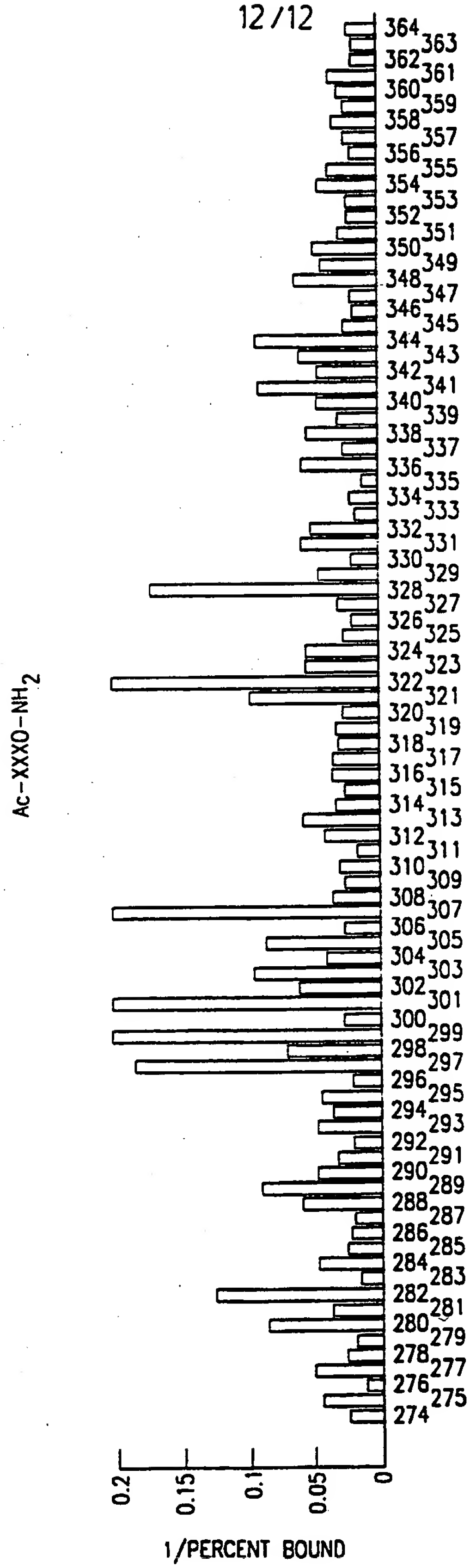


FIG. 11C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279
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276
275
274
0.2
0.15
0.1
0.05
0
1/PERCENT BOUND

FIG. 11D

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US98/03298

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :A61K 38/00, 38/02; CO7K 5/00, 7/00
US CL :530/329, 330; 514/16, 17, 18
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 530/329, 330; 514/16, 17, 18

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

LIBRARY CAS ONLINE, APS, BIOSIS, EMBASE, MEDLINE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y,P	US 5,726,156 A (GIRTEN et al.) 10 March 1998, see entire document.	1-25
Y	US 5,618,791 A (DU) 08 April 1997, see entire document.	1-25
Y	WO 97/22356 A1 (HOUGHTEN PHARMACETICALS, INC.) 26 June 1997, see entire document.	1-25
Y	HASKELL-LUEVANO ET AL., Discovery of Prototype Peptidomimetic Agonists at the Human Melanocortin Receptors MC1R and MC4R. J. Med. Chem., 1997, Vol. 40, No. 4, pages 2133-2139, see entire document.	1-25

☒ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

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"A" document defining the general state of the art which is not considered to be of particular relevance

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"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
03 AUGUST 1998	08 SEP 1998

Name and mailing address of the ISA/US	Authorized officer
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Form PCT/ISA/210 (second sheet)(July 1992)★

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US98/03298

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	SCHIOTH et al., Characterization of the binding of MSH-B, HP-228, GHRP-6 and 153N-6 to the human melanocortin receptor subtypes. Neuropeptides. 1997, Vol. 31, No. 6, pages 565-571, see entire document.	1-25